Niranjan Bhattacharya
Phillip G. Stubblefield
Editors

Human Fetal Growth and Development

First and Second Trimesters

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The book is dedicated to two scientists working on two sides of the globe. They are both no more but their work lives on. They were not connected to each other, had never met each other and worked in very different fields within the broad parameters of fetal growth, abortion, and continuation of pregnancy; yet they are connected by their honest and sincere dedication to scientific research.

Prof. A E Beer of the University of Michigan, USA, is the father of the "Beer Baby." He was one of the best known Reproductive Immunologist in the world during his lifetime. From 1977 till his death he had regular correspondence with Dr. Niranjan Bhattacharya, then a young scientist, a correspondence that spanned thousands of questions raised by Dr. Bhattacharya and answered patiently and in great depth by Dr. Beer. Once Dr. Bhattacharya went to meet him in 1986 at Ann Abor and after a talk of over two hours felt that Dr. Beer was not his usual self. He queried him about this, and Dr. Beer's answer reflects the man he was and his dedication to science: "I am sorry but my brother is now undergoing corrective operation for the dissecting aneurism of the aorta, but I had given you an appointment…"

Prof. Chameli Ganguly was a Physiologist-turned-Biochemist. She started her career as a Junior Research Fellow under Prof. Kanailal Mukherjee, a legendary biochemist who worked in the Institute of Post Graduate Medical Research, Calcutta, India. She was married to science. She considered any manipulation of scientific data as a rape of science and would often repeat an experiment 20 times with immense patience if the need arose. She is instrumental in supporting this book from the conceptual phase because she believed that

it was important to globally disseminate the findings of her and others' work in this very challenging field of fetal growth and development, especially to understand why the fetus survives or why it is sometimes expelled.

The editors dedicate this book to Dr. Beer and Dr. Ganguly. May they motivate many more to follow in their footsteps.

Prof. Niranjan Bhattacharya, Kolkata, India
Prof. Phillip G. Stubblefield, Boston, USA

Preface

Omh Ayamaramva sybhya bhabatu (Sanskrit)
Let this effort be for the welfare of all.

I met a little Elfman once,
Down where the lilies blow,
I asked him why he was so small,
and why he did not grow.
He slightly frowned and with his eyes
He looked at me through and through.
"I'm quite as big for me", he said
"As you are big for you".
J.K. Bangs

In *Scientific Foundations of Pediatrics* edited by J.A. Devis and J. Dobbing, William Heinemann Medical books Ltd., 1981 (2nd edition).

Poets are visionaries. They can see beyond themselves. He has expressed exactly what I want to say. The growth and development of a human fetus within the mother's womb is conditioned by the genetic makeup and the micro and macro environment provided by the mother.

Back in 1964, Prof. K L Mukherjee undertook a project for the anthropometry of human fetuses. Much information is available about the growth and development of human babies after parturition but because of the inaccessibility of human fetuses in intrauterine life not many studies are available on anthropometry of fetuses. In 1964, when the medical terminations of pregnancy clinics were started, Prof. K L Mukherjee had the opportunity of studying the anthropometry of human fetuses at different periods of gestation.

At that time, ultrasonography was not available in all hospitals. The studies also include the structure and some functional aspects of individual organs. Multiparous mothers undergoing the operation of hysterotomy and ligation at the same time at the Medical Termination of Pregnancy clinic were the subjects of the present investigation. The project was explained to the mothers who signed the consent form for the use of the fetuses. The project was duly cleared through the Ethical Committee of the Institute. The usual modus operandi was as follows:

Anesthetized mothers preoperatively received an injection of ergometrine. An incision was made above symphysis pubis. The uteri were cut through usual steps and whole fetuses including the intact amniotic sacs were taken out with the help of fingers and placed on an ice bucket. The fetuses were brought to the laboratory and dissections were started within 45 min.

The fetuses were weighed to the nearest milligram. The crown heel and crown rump length were measured to the nearest millimeter with a tape measure. The biparital diameter at the level of the eyebrow were measured with a thread and the length was calculated from the length of the thread.

A total of 1396 fetuses were studied during the period 1964–2007. Some details of anthropometric measurements are available in Langman's Medical Embryology and Morsman's Human Embryology. Baslinsky's book on embryology was a source of many informations. The reasons which prompted us to publish the results were the large number of fetuses which were available to us.

Besides anthropometry, attempts were made to study the histological structure and some of the characteristic biochemical functions of individual organs of the fetuses. The following organs were specially studied: liver, lungs, brain, intestine, kidneys, gonads, thymus, adrenal cortex and skeletal muscle. Since our limitations for the hysterotomy extended up to 20 weeks only, most of our studies were limited to that period of intrauterine life. The gestation periods were calculated from the last menstrual period. Only a few fetuses of older periods were available for studies; all of them were stillborns.

The mothers were all normally nourished women and did not have any sign of under nutrition. It is presumed that all the fetuses were fully nourished for their periods of gestation. None of the mothers had anomalies of placenta. The idea behind the study is to understand the growth of the individual fetus along with its development and maturation of all the organs.

Growth implies both cell multiplication and accumulation of both intra- and extracellular material. It seldom involves just cell multiplication and matrix secretion, but simultaneously it causes cell differentiation, pattern formation, and changes in form. In case of normal development, a great range of functions is simultaneously orchestrated to produce a harmonious pattern which is characteristic of normal development. The growth and maturation of an organ at the cellular level comprises of four stages, viz., (1) proliferation, (2) migration, (3) differentiation, and (4) death.

From fertilization, the embryogenesis period continues up to the tenth week of gestation. The fetal period begins at the end of the tenth week of gestation (eighth week of development). Since the precursors of all the major organs are created by this time, the fetal period is described both by organ and by a list of changes by weeks of gestational age. Because the precursors of the organs are formed, the fetus is also not as sensitive to damage from environmental exposure as the embryo. Instead, toxic exposures often cause physiological abnormalities or minor congenital malformation.

There is much variation in the growth of the fetus. When fetal size is less than expected, that condition is known as intrauterine growth restriction (IUGR), also called fetal growth restriction (FGR). Factors affecting fetal growth can be *maternal*, *placental*, or *fetal*. Maternal factors include maternal weight, body mass index, nutritional state, emotional stress, toxin exposure (including tobacco, alcohol, heroin, and other drugs which can also harm the fetus in other ways), and uterine blood flow. Placental factors include size, microstructure (densities and architecture), umbilical blood flow, transporters

and binding proteins, nutrient utilization, and nutrient production. Fetal factors include the fetus genome, nutrient production, and hormone output. Also, female fetuses tend to weigh less than males at full term.

Fetal growth is often classified as follows: small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA). SGA can result in low birth weight, although premature birth can also result in low birth weight. Low birth weight increases risk for perinatal mortality (death shortly after birth), asphyxia, hypothermia, polycythemia, hypocalcemia, immune dysfunction, neurologic abnormalities, and other long-term health problems . SGA may be associated with growth delay, or it may instead be associated with absolute stunting of growth.

Kolkata, India Kanailal Mukherjee
Kolkata, India Niranjan Bhattacharya
Jamaica Plain, MA, USA Phillip G. Stubblefield

Introduction

Research studies on the growth and development of the human fetus, an area of study that has important implications for the development of a human being, and possible diseases that the adult human may face later in life due to problems suffered in utero, are few. This is understandable because while studies on animal models are easier to conduct with fewer ethical restrictions, research on human fetuses carries with it inherent difficulties and complications. However, with the development of new technologies, imaging and other ways of monitoring growth have become easier and safer. Further, medical termination of pregnancy for different purposes and reasons are permitted in several countries, providing the opportunity for study and research after obtaining informed consent from all concerned. The present book is an attempt to bring together knowledge in this very significant field from all over the world under the covers of one book.

The present project, which now sees fruition in a published form, actually started a long time back as an experimental effort to study the growth and development of the human fetus in utero after parturition by a group of researchers, senior and junior, in Kolkata (then known as Calcutta) under the leadership of a senior professor, Dr. K L Mukherjee. As mentioned, due to the inaccessibility of human fetuses in intrauterine life, not many studies are available on the anthropometry of fetuses. In 1964, medical termination of pregnancy clinics were started by Prof. K L Mukherjee at the Institute of Post Graduate Medical Education and Research (IPGMER), a premier research institution situated in the SSKM Hospital, Calcutta. The setting up of the clinic in SSKM Hospital provided the opportunity for studying of anthropometry of the human fetus at different periods of gestation on the aborted fetuses. The Medical Termination of Pregnancy Act was passed in 1971; this specifies, among other things, that any pregnant woman, within the prescribed weeks, can legally request her pregnancy to be terminated in selected government hospitals, equipped for the purpose, without legal harassment, for the purpose of family planning. The study of fetuses became much easier after the passage of this Act, since mothers admitted for hysterotomy and ligation, if they gave informed consent and the Ethical Committee gave clearance, were taken as subjects and their fetuses were collected from the operation theater of government hospitals only, for study and research.

And the ball started rolling. On the 50th year of our continued research, we decided to publish our findings through an international book project on the growth and development of the human fetus up to the second trimester.

The person who had initiated the idea of publishing this book, which would also cover research on the subject done all over the world, Prof. Chameli Ganguly, a sincere researcher who had been part of the team since the beginning, recently passed away. We expedited the entire process, convinced that the fruits of our research should not be lost but shared with future generations working in this and related fields, particularly since there is so little in-depth work in this very important area with its manifold implications. The global giant in medical publication, Springer International, was convinced of the importance and hence this book.

When we began our study in the mid-1960s, the criteria for prematurity was (1) babies born before term, or (2) weighing less than 2500 g at birth. However, the situation has changed over the last 50 years of development of medical science.

Currently the world's smallest premature baby to have survived from birth until hospital discharge was born 15 weeks before its due date (born: September 4, 2004). At birth, the baby weighed just 260 g, or 8.6 oz. She was 9.8 in. long. Her mother suffered from severe pregnancy-specific gestosis. This tiny little girl became clinically normal at 7 years, though she had to undergo laser eye surgery for retinopathy of prematurity (ROP).

This is not an isolated case. The second smallest baby in the USA and the world's third smallest baby to survive long enough to leave the hospital was born at 24 weeks because her mother suffered from similar pregnancy-specific hypertensive disorder. She weighed just 9 ½ oz at birth. Supplemental oxygen was given to her at home to treat bronchopulmonary dysplasia (BPD), and she had to have surgery to repair a PDA and laser eye surgery for ROP. Her brain was free from any bleeding.

Another interesting case is the birth of a boy in Germany, who was delivered in June 2009 at 25 weeks gestation. He weighed 275 g, just over 9 ½ oz. He wears glasses and needed physical therapy during his first year of life, but is otherwise a normal, healthy toddler. Another girl baby was born in 1989, who became the world's sixth smallest surviving preemie. She weighed just 280 g at birth (9.9 oz) at 26.6 weeks gestation. She is only 4 ft 7 in. tall, wears glasses, and has asthma; she has no other long-term effects of her premature birth.

The record for the world's most longest surviving (till now) premature baby belongs to James Elgin Gill, a Canadian male born at just 21 weeks 5 days in 1988. James beat all of the odds, and in 2006 was a healthy teenager heading off to college. Another American baby girl was born at 21 weeks 6 days in October of 2006 via in vitro fertilization; her gestational age can be pinpointed exactly, an impossibility for most infants. Although she needed oxygen at hospital discharge, was anemic, and has mild osteopenia, she is otherwise a normal, healthy little girl. The babies mentioned above show no major developmental delays. However, the outcomes for micropreemies are not always as good.

The present book started with an attempted understanding of a basic issue, what is fetal growth?

This issue has been examined from various angles, starting with discussions on the anthropometric measurements of infants born at various

gestational periods which are an indicator of gestational age (Chap. 1), the use of developmental immunology to understand the immunological system of a fetus and explain why a newborn is vulnerable to many infections (Chap. 3), debates regarding the vaccination of the unborn (Chap. 2) and tracing the actual growth of a fetus from the zygote stage through the second semester (Chap. 5), to the search for a safe, effective, mid-trimester abortifacent, which involved the use of antigens to disrupt the growth of the fetus and which has revealed many hitherto unknown secrets about the growth and development of the human fetus up to 20 weeks (Chap. 4).

The book goes on to particulars from this basic issue and delves into the complexities of growth, structural and functional development, carbohydrate metabolism and urea biosynthesis in the human fetus, development of the fetal brain and the role of lipid metabolism in human fetal growth. Chapter 6 suggests that growth of individual organs in a fetus is conditioned by the genetic potential and the environment provided by the mother and a third component, the fetus itself. The importance of maternal circulation in the supply of glucose to the developing fetus is highlighted in Chap. 7, and Chap. 8 studies the appearance of mucopolysaccharides, water, and electrolytes in developing human fetal organs, namely, the liver, lungs, and brain, and finds that in all three the water content is much higher in fetal life than in adult life and that there is slow decrease in the water content with the progression of development. Chapter 9 examines the biosynthesis of urea in the fetal liver up to the second semester: one of the various findings was that very little urea was synthesized by the fetal liver, especially in the earlier period of gestation; the rate increased with progress of gestation but in comparison to the situation in an adult, very little urea was synthesized even in the later periods of the gestation. It was further found that of the two possibilities of utilization of ornithine besides the urea cycle, the proline biosynthesis and polyamine biosynthesis, the former pathway was found to be absent in fetal livers. Chapter 10 raises and tries to answer an interesting question: can the fetal and adultogenic layer be distinguished by any other characteristics? Since the functional characteristics of the two kinds of cells also differ, can we identify any protein or other component present in the fetal layer of cells which is absent in the adult layer? The researchers, from their experiments, found that a set of antigens were found in adult extracts which were not present in fetal extracts. The researchers inferred that either the adult saline extract does not have some common fetal antigens or the differentiation of the adult adrenal gland is associated with the appearance of a host of specific antigens. Chapter 11 traces the growth of the human fetal brain up to 28 weeks of gestation and finds that there is a straight-line relationship between the body weight and brain weight. The weight of the brain of fetus is around 14 % of the body weight throughout the gestation period. The experiments also suggested that in the case of brain, some acid soluble nucleotides are very importantly and significantly involved in myelin synthesis and there may be higher metabolic activity and turnover at earlier periods. Chapter 11 discusses lipid metabolism in human fetal development. The authors note that the fetus requires a substantial amount of lipids throughout its development; the lack of lipids including cholesterol (CHO) affects growth disorders. The two lipoproteins

(LPs) involved in supporting the placental CHO need are low density lipoprotein (LDL) and high density lipoprotein (HDL). Fetal steroid precursors of estrogens regulate the uptake of maternal LPs to promote the placental progesterone synthesis. Both estrogen and progesterone are key determinants in pregnancy maintenance and fetal growth; this is a basic role of fetal and maternal LPs.

The next seven chapters study various implications of genes on fetal development up to the second trimester. Chapter 13 is a study of gene regulatory networks and epigenetic modifications in cell fate decisions during early embryonic development. Cell differentiation takes place as the fertilized totipotent cell progresses along the developmental pathway. The authors discuss gene regulatory networks and epigenetic modifications in early development as regulation of expression of genes is the key to understanding how cell differentiation occurs in early developmental stages. The author also discusses master regulators involved in differentiation of the trophectoderm and the inner cell mass, which are incompletely known and the chromatin modifiers involved in these steps. Further, he also describes the master regulators that control the cell fate decisions that give rise to the epiblast and the primitive endoderm from the inner cell. Chapter 14 is a different kind of chapter; it explores the possibilities of finding out when an embryo reaches "personhood." This has major implications for fetal stem cell research because the generation of human embryonic stem cells sometimes requires the destruction of early human embryos, and ethical questions are raised about stem cell research. Chapter 16, by the same author, is another theoretical exploratory chapter on the embryological basis of virgin birth. Chapter 15, by the same author, discusses dependence of fetal hairs and sebaceous glands on the fetal adrenal cortex and the possible control from epidermal Merkel cells and adrenal medulla. The role of the fetal adrenal cortex is little understood. This chapter reviews the literature and attempts to synthesize the current understanding of the developmental and functional biology of the fetal adrenal cortex. Chapter 7 discusses aneuploidy in human preimplantation embryos. The author notes that the number of abnormal zygotes cannot simply be calculated on the basis of the aneuploidy rates of gametes but may result from abnormal fertilization. Furthermore mitotic abnormalities during cleavage division give rise to mosaic chromosome abnormalities of the embryos. Chapter 17 throws new light on the potential impact of maternal milk consumption during pregnancy on mTORC1-driven fetal growth. The author notes that milk is a highly specialized, complex signaling system developed by mammalian evolution to promote mTORC1-driven postnatal growth but milk is not designed by nature to promote prenatal fetal growth. Continued consumption of cow's milk and dairy products during adolescence, adulthood, and pregnancy is an evolutionarily novel behavior that may have long-term adverse effects on human health. The authors of Chap. 19 present their findings on genetic disease specific human embryonic cell lines. They have detected affected embryos, which were used for derivation of the genetic disease specific hESC lines, which presently contain 87 hESC lines obtained from embryos with single-gene and chromosomal disorders. Further, screening of 137 normal hESC lines for polymorphism in the chemokine receptor 5

(CCR5) CMKBR5 gene resulted in detection of 12 hESC lines with CCR5-del32 allele, including one with two copies of the gene, conferring resistance to HIV.

The next section of the book discusses the impact of stem cells on the growth and development of the human fetus. Stem cells are considered the origins of all organisms. In fact, all organisms are formed and developed from a single cell – the zygote, which is the product of oocyte fertilization by the sperm. Through the process of cell division, the totipotent stem cell produces all tissue types within an organism. By means of self-renewal, stem cells are maintained during embryonic development right through to adulthood. The basics of role of stem cells in the development of the human fetus are discussed in Chap. 20. The next chapter discusses how and possibly why a human fetus survives in utero without being rejected by the mother (Chap. 21). This involves the mother's immune system. The authors suggest that during a successful pregnancy, the immunity that may cause rejection of the baby and placenta is shut off in the mother's uterus. Throughout the rest of the mother's body, her immune system is fully functional, allowing her to deal in a proper way with any infections that may come her way. In cases of recurrent miscarriage, this immune camouflage and protection of the embryo is perhaps not initiated in such a way that the uterine local and systemic immune responses of the mother are modulated in favor of a decrease in cell mediated immunity and increase in humoral immunity.

The next few chapters go on to discuss the development of different systems like the immune system, the endocrine system, the hepatic system in the human fetus and its impact on growth and maturation. Chapter 22 focuses on glucose metabolism in the fetus and its relationship with fetal insulin. The regulation of glucose, the main energy source of a fetus, is kept fairly constant by the maintenance of maternal glucose concentration by increasing rates of maternal glucose production and development of relative maternal glucose intolerance and insulin resistance, and secondly, the transfer of maternal glucose to the fetus by the placenta, which is buffered by placental glucose utilization. A third regulating factor is the production of insulin by the developing fetal pancreas, which enhances glucose utilization among the insulin-sensitive tissues (skeletal muscle, liver, heart, adipose tissue) that increase in mass and meet the glucose need during late gestation. Chapter 23 notes that systemic maturation is essential for the survival of a neonate, and focuses on the maturation of the endocrine system which is necessary to generate hormones to support normal development. A bouquet of transition factors and epigenetic events act hand in hand with autocrine, paracrine and endocrine network of hormones and growth factors leading to the evolution of the fetal endocrine system with the hypothalamus-pituitary system providing the pivotal controlling template. However, most of the evidence in this chapter, the author mentions, comes from animal studies. The next chapter (Chap. 24) discusses development of the human immune system in the first and second trimesters. The immune paradox lies in the fact that the semi-allogenic embryo (or allogenic embryo in surrogate mothers) is not immunologically rejected. She notes that the fetal immune system can tolerate antigens (including its mother and its own organs) better than to eliminate

antigens from its environment. Trophoblast-derived thymic stromal lymphopoietin (TSLP) instructs decidual CD11c+ dendritic cells (dDCs) with increased costimulatory molecules, MHC class II, and Th2/3-type, but not Th1-type, cytokines. TSLP-activated dDCs cause proliferation and differentiation of decidual CD4+ CD252 T cells into CD4+ CD25+ FOXP3+ regulatory T cells (Tregs) through TGF-b1. Decidual CD4+ CD25+ FOXP3+ Tregs promote invasiveness and HLA-G expression of trophoblasts, resulting in preferential production of Th2 cytokines and reduced cytotoxicity in decidual CD56brightCD162 NK cells.

The next few chapters focus on fetal hepatic development (Chaps. 25 and 26), fetal cardiovascular development (Chaps. 27 and 28), fetal neurological development and its implications on diseases on adult life (Chaps. 29 and 30), fetal nephrological development (Chap. 31), and fetal hematological development (Chap. 32). The proper development of the fetal liver, heart, and all other organs is vital to later well being. The liver is indispensable for maintenance of normal homeostasis. The development of the liver is a complex process involving an intricate web of various transcription factors which interact among each other and are regulated by extracellular signaling (Chap. 25). The liver is also the abode for a variety of stem cells – both for the bone marrow derived, which are pluripotent and home to the liver, as well as the hepatic progenitor cells (HPC), which are more differentiated, oval cells – playing roles in hepatocyte regeneration and response to injury (Chap. 26). Understanding the process of development of the liver may help in the creation of new cell therapies: growing tissues ex vivo for use in transplantation or for coaxing cells in vivo to acquire characteristics that can restore function in disease states. So far as the heart is concerned, abnormal embryonic development of the vascular system causes a variety of vascular anomalies. Vasculogenesis is genetically determined with a specific sequence of formation, selective regression and remodeling that must occur in the correct sequence to produce the typical mature vascular system. Investigation into the genes and signaling pathways that guide this complex process are crucial to understanding how vascular anomalies develop and may ultimately provide clues for therapeutic intervention or treatment of these conditions (Chap. 27). However, modern imaging technologies provide hope for structural cardiac defects (Chap. 28). Once the structural cardiac defects are identified by an obstetric sonologist, the precise details of CV defects are studied by a fetal echocardiographer. In selected subsets of fetuses, further details may be gathered with other imaging modalities. Finally, a detailed treatment policy can be formulated well before the baby is born. Fetal growth and development also has an impact on adult neurodegeneration. Chronic placental insufficiency may result in fetal hypoxemia which leads to synaptic dysfunction that triggers neurodegeneration in neonates. Maternal hormonal disturbances also affect the fetus adversely (Chap. 29). However, there are few human studies showing the effect of in utero exposure on neurodegeneration. The next chapter has collected data on low birth weight babies and observes that for over 50 years, preterm outcome studies have reported higher rates of cerebral palsy, intellectual disability, and sensory impairment in preterm survivors as compared with peers born at term. Chapter 31 discusses the development of

kidneys in fetuses up to the second trimester, and claims that this organ is the "most important organ evaluated in nature." Chapter 32 examines fetal hematological development, noting the various stages of development and observing that like primitive erythropoiesis in the yolk sac, definitive erythropoiesis in the fetal liver is essential for continued survival of the embryo.

The next three chapters evaluate the pharmalogical implications of fetal development and the liabilities of treating the fetus as a patient. Chapter 33 discusses twin-twin transfusion syndrome and perinatal mortality. However, overall rates of perinatal survival have enhanced as a consequence of a scope of treatment modalities, including amnioreduction, septostomy, and laser ablation. Amnioreduction offers great results in right on time stage sickness, with at least one fetus surviving in more than 85 % of cases and two surviving in 66.7 % of cases with stage I or stage II disease. Chapter 34 also notes that previously treatment was restricted to the neonatal period, but with recent advancements in the basic science of medicine and surgery, there is a high possibility to detect and treat any anomaly of the fetus prenatally. Chapter 35 focuses on structural and functional perspectives on the placental barrier and its role in fetal development in the first two trimesters. The authors note that the placenta plays a significant role in providing a micro niche which supports the fetal growth through transfer of nutrients and immunological properties which helps in imparting protective immunological properties to the fetus. It also acts as a major fetomaternal barrier against pathogens from the maternal system. It also undertakes the secretion of different hormones, cytokines, growth factors, and other bioactive products essential for the fetus.

Chapters 36 and 37 explain surgery on the unborn. This is a relatively new field and is still developing. Surgeries are being undertaken for several anomalies like congenital diaphragmatic hernia, thoracic malformations, twin–twin transfusion syndrome, airway obstruction, aortic valve stenosis, and myelomeningocele. The first chapter reviews prenatal process and a variety of operating approaches for fetal surgery, while the second discusses an experimental therapy involving maternal laparotomy and hysterotomy, delivering the fetus and performing the necessary fetal surgery, after which the fetus is returned to the uterus and the pregnancy allowed to continue. The author observes that at subsequent delivery, there was a lack of scarring and contracture associated with the surgery. While this makes the procedure attractive, there are risks involved in interrupting a healthy and viable pregnancy. He notes that perhaps more relevant, at this stage, is investigating the biology of fetal wound healing to discover the mechanisms of scarless healing.

Chapter 38 focuses on the relation between intrauterine growth retardation (IUGR) and medical complications in adult life. Gross IUGR can lead to hypertension, increased risk of metabolic like dyslipidemia, diabetes mellitus, and cardiovascular disorders, renal impairments in some cases, improper brain and neurofunctional development and, in some rare cases, onset of auto-immune diseases also like rheumatoid arthritis and systemic lupus erythematosus.

The following six chapters are rather unique and provide very different perspectives on human fetal growth. There are many streams of alternative

medicine, and the fact that they have survived over hundreds of years and sometimes millennia means that they cannot be ignored and that modern medicine should incorporate scientific truth from wherever it can be found. Chapters 39 and 42 provide perspectives on fetal growth and development from the point of view of traditional Chinese medicine, which also has potential for therapeutic remedies for threatened miscarriages. Chapter 40 gives an indication on how tribal people in India can predict the sex of a baby by measuring the circumference of the belly of a pregnant woman during the second trimester and observing fetal movement. Tribal medicine also prescribes nutrients and discourages some kinds of food and drinks during pregnancy for the proper development of a fetus. The next chapter (Chap. 41) discusses applications of Unani medicine in pregnancy, though it is generally considered unscientific in the West. Homeopathy (Chap. 43), although the invention of a German scientist, is also discarded by the West as a non-viable science, though it has helped many mothers through difficult pregnancies. Chapter 44 evaluates the potential of Ayurvedic medicine in the first two trimesters of pregnancy.

Finally, the book also contains some articles which cannot be labeled in the categories mentioned above. Chapter 45 delves into the problems of prematurity. It describes the short-term and long-term complications of preterm birth in terms of fetal development as well as injury to fragile organ systems during the perinatal and neonatal periods. Although some randomized, controlled trials demonstrate the safety and effectiveness of a few treatments for neonates, scientific neonatal intensive care unit (NICU) treatments and interventions have not been adequately investigated. Therefore, the author recommends more rigorous studies of new therapies and medications before they are adopted. The next chapter (Chap. 46) also discusses the problems of extreme prematurity and notes that preterm birth is the most common cause of death among infants worldwide, and that the chances of survival without long-term difficulties is low. Chapter 47 focuses on the idea of sequencing the naturally aborted fetuses and its potential biological implications. The author believes that such a resource can easily be generated in India and such a database can provide answers to many crucial yet unsolved problems related to premature delivery to rejection of the fetus. This is also likely to answer several developmental issues that decide the progress from successful fertilization and fetal development to a fully mature baby. The final chapter (Chap. 48) pertains to the ethics of the use of aborted human tissues for research and therapeutic purposes. The book would not be complete without this chapter since ethics is an intrinsic part of medical research.

The editors gratefully acknowledge all the contributors to this volume. They have not been individually named here; instead, the editors have tried to highlight the multiple themes that have been discussed in the book. But it is essential at this point to say that contributors from all over the world – the Chinese University of Hong Kong; Yale University School of Medicine; the University of Science, Vietnam National University; Reproductive Genetic Institute, Chicago; University of Osnabrück, Germany; Maastricht University Medical Center, the Netherlands; Gulhane Military Medical Academy, Ankara, Turkey; Department of Health Science and Pediatrics, Turin

University, Italy – participated in this book project. The book also has a large number of contributors from premier Indian medical research institutions and other scientific institutions. The book, while beginning with the basic work done by Prof. Kanailal Mukherjee and his team, encompasses other research and theoretical perspectives and speculations from researchers across India. The editors have tried to make the book as comprehensive as possible, particularly because published works in the area of human fetal growth, development, and maturation are few and far between. Yet this is an area of growing importance, given the emergence of stem cell biology and regenerative medicine as an increasingly significant discipline because of the new potentials of cure and therapy that it promises. However, without understanding the growth and development of the human fetus, especially in its early stages, it will not be possible to understand how stem cells can help in regeneration of damaged tissues. The editors are therefore thankful to all the contributors for sharing their knowledge in this book. However, needless to say, the editors bear no responsibility for the views of the contributors, which are the products of their study and research and, therefore, their own.

Prof. Niranjan Bhattacharya and Prof. Phillip G. Stubblefield

To all scientists – present, past and future – whose sincere dedication to science and the search for truth has made science into a religion

Dr. Niranjan Bhattacharya, Kolkata, India
Prof. Phillip G. Stubblefield, Boston, USA

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We would like to express our gratitude to the many people who saw us through this book; to all those who provided support, talked things over, read, wrote, offered comments, allowed me to quote their remarks and assisted in the editing and design.

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Part I

Introduction

Fetal Growth

1

Niranjan Bhattacharya and Phillip G. Stubblefield

Introduction

Prenatal studies of commonly used research animals do not provide good models for fetal growth patterns. Studies of human intrauterine growth usually are based on anthropometric measurements of infants born at various gestational periods. Gestation is usually estimated in weeks from the first day of the last menstrual period before conception. Thus, conceptual age and the period of actual embryonal and fetal growth may be 1–3 weeks less than the estimated gestational age. Numerous centile charts have been constructed which relate fetal measurements to gestational age [1, 2]. Studies of birth weight in relation to gestational age have been carried out in large population samples usually on a retrospective basis. They have emphasized that maternal height, weight gain in mid-pregnancy, parity, age, ethnic group, genetic factors, socio-economic factors, nutrition before and during pregnancy and smoking during pregnancy, all may influence fetal growth.

Fetal growth like post-natal infant growth undoubtedly is influenced by nutrient availability for mother and fetus and nutritional state of the mother before and during pregnancy. The nutritional state of the mother when she was herself a fetus may also be a significant regulator of the growth of her fetus [3].

The maximal velocity of fetal growth appears to be between 32 and 38 weeks of gestation. During that interval the birth weight virtually doubles. By 39 weeks the peak growth is reached. Normal full term gestation is considered to be between 37 and 41 weeks. If born before 37 weeks, the infant is considered premature. If born after 41 weeks it is considered post-mature. Mean birth weight for babies are smaller in most developing countries than in Europe, the United States of America and Canada. This may be due to a shorter period of high velocity of growth, perhaps related to maternal nutrition prenatally and during pregnancy.

Physical Growth and Its Measurements

Although growth is a highly complex process, it takes place in a completely ordered fashion in the biological system. The intrauterine period of mammalian growth can be divided into three

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distinct phases, in each of which the mode of nutrition is different [4]. In the first blastocyst phase, nutrition is derived from the uterine secretions. In the second or embryonic phase, nutrition is derived from the active erosive agency of the fetal trophoblast, while in the third fetal phase, trophoblastic activity ceases and nutrition is derived by diffusion from the maternal blood stream.

EM Widdowson [5] described the changes in the body composition of the fetus during growth. The initial division of the fertilized ovum is not accompanied with an increase in its size, and this may be true for a few further divisions, so that each cell becomes progressively smaller and there is no increase in total size. By the time the blastocyst has become implanted in the wall of the uterus, about the ninth day after fertilization, synthesis of proteins from the amino acids, and water and inorganic substances that reach it, begin to enlarge the cells before they divide. There is an alteration too in the timing of the divisions. The first few occur almost simultaneously, but after a few days, cell division becomes staggered so that only a few cells are dividing at the same time.

The immature organism is characterized by the large percentage of extracellular fluid within it. When the ovum is fertilized and for the first few generations of divisions, the new organism is entirely cellular, so there must come a time in early development when extracellular fluid becomes a part of it. There is an upper limit to the size of a cell that it can attain. This is partly because as it grows, its volume increases with the cube of the radius but its surface area expands with the square. The volume determines its biochemical activity, but the materials necessary for this activity must pass in through the surface membrane. Further, as the cell increases in size the ratio between the cytoplasm and the nucleus increases, and there is again an upper limit to the amount of cytoplasm that the nucleus can control.

In the first 2 or 3 weeks of human development inside the uterus, growth takes place entirely by cell division. Differentiation soon begins and as the organs and tissues appear, each develops its own characteristic kind of material and its own contribution to the weight of the organism as a whole. With differentiation comes a slowing down of cell division and as this occurs the average size of the cells begins to increase. This is accompanied by a progressive increase in the proportion of organs occupied by cells and a decrease in the percentage of extracellular fluid. This process of decreasing of extracellular fluid goes on during fetal and early postnatal life [6].

As an animal grows, its body proportions and composition change. These alterations are brought about by a differential rate occurring in the different parts and tissues of the body [7]. The order in which the various parts and tissues develop is much the same in all species, for it is based on the relative importance of the functions of the parts or tissues for the survival of the animal.

The changes in the form of embryo as it develops, repeat in general, the evolution of the species [8]. In horses, cattle and sheep, whose young are born at an advanced stage of development and have to follow their mother, the maximum proportions of leg length occur at birth, while in rabbits, which are born at a less advanced stage of development, this does not occur until later. In general, the head and the legs form a high proportion of the body weight at birth. With development, the body first lengthens and later deepens. The growth gradient starts at extremities and passes inward to the loin, while the lower parts of the ribs are the latest to develop.

When growth is limited by a low level of nutrition, the earlier developing parts and tissues have priority of supply [9].

The growth of the body and the changes in its composition during development may be considered in a number of different ways. Growth and development begin at the moment of conception and are continuous from then on until the epiphyses fuse and growth in height ceases, and the processes concerned with chemical development come to an end. Data on growth and composition of the body before term at 40 weeks gestation are of necessity cross-sectional. Measurement of growth in size after births are mainly longitudinal, but measurement of body composition at all ages has not been longitudinal [6]. Before describing early growth it is necessary to consider briefly the measurements of growth which are available in clinical practice, i.e., measurement of physical growth.

Weight

Weight is still the most widely used single clinical measurement of growth in intrauterine and postnatal life. Weight can be regarded as the sum of body fat and lean body tissue so that weight gain represents the sum of increments in different body components including muscles, skeleton, adipose tissue and water. It is therefore a non specific measurement of growth. In order to provide a more detailed assessment, other measurements are needed and crown-heel length, head circumference, biparietal diameter, etc. are now being increasingly used. The velocity of the growth of the body in weight and length is more rapid during the last 3 months of gestation and the first 4 or 5 months after birth than at any other age [6].

After birth the internal organs – heart, kidneys, lungs and liver – tend to grow parallel within the body, and they form roughly the same percentage of the body weight in all ages. Data based on P Gruenwald and HN Mainh [10] and E Boyd [11] show that before term, most organs contribute a little more to the weight of the body than they do during childhood and adolescence, but the spleen stays constant at 0.3 % of the body weight from 26 weeks gestation to 13 years.

Length

The accurate measurement of crown-heel provides the best clinical measurement of skeletal growth since, unlike weight gain, it is not influenced by the accumulation of water and fat [12].

Head Circumference

Indirect measurements have been used to follow the normal growth of the human brain. The most common of these is cranial circumference. Some correlations have been made between increase in cranial circumference and the cellular growth of the brain. Approximate formulae have been worked out relating head circumference to brain weight, protein and DNA content during the first year of life [13].

Nucleic Acids

In the rat, DNA synthesis and hence, cell division cease in the brain 20 days after birth [14]. Net protein synthesis continues to about 99 days of age and lipids are synthesized for varying periods between these extremes. Thus, the total number of cells is reached before the total protoplasmic mass of the brain is attained. There is a phase during which already existing cells must be enlarging in order to account for the over-all enlargement of the brain. Actually, careful examination of all non-regenerating organs reveals three distinct phases of growth. The first stage of growth is characterized by a proportional increase in weight, protein and DNA content. The number of cells is increasing whereas the ratios of the size of the individual cells are not changing. Simple hyperplasia in this case is occurring. This phase ends as the rate of net DNA synthesis begins to slow down and weight and protein content continue to increase at the same rate giving rise to a transitional phase of hyperplasia and concomitant hypertrophy which lasts until net DNA synthesis stops. After this point, all growth is by hypertrophy. Finally, when weight stabilizes and net protein synthesis stops, growth is finished. The ultimate packaging of the protoplasm into individual cells depends on the rate of DNA synthesis. The time during the growing period when cells are actively proliferating in any organ is presumably under genetic control and these times are different in different species. For example, in the rat, cell division in the brain is over by 21 days postnatally. In the guinea pig there is very little cell division in the brain after birth [15]. In contrast, in the human, cell division in the brain continues until around the end of the first year of life [16]. The enzyme DNA polymerase is essential for the synthesis of DNA. It has been shown that the activity of this enzyme mimics the rate of DNA synthesis in the chick brain [17] and in the rat brain. In the rat, the maximum rate of DNA synthesis is achieved at around 10 days of age. Peak enzyme activity is attained at 6–10 days of age and the activity is highest in those regions where cell division is most rapid [18].

EM Widdowson et al. in 1972 [19] examined cellular growth in the human fetal liver as judged by measurements of DNA and protein. They found that total DNA increased rapidly from around 15 weeks until term. The protein DNA ratio however was virtually unchanged from 15 to 25 weeks, suggesting that during the early fetal stages, the cell number is increasing more rapidly than the cell size. Also, some lipid components (diphosphoinositide, sphingomyelin, cerebrosides etc.) are absent in the early stages of individual development and appear only in the last stage of fetal development or in the first weeks of postnatal life [20].

Proteins

Development and metabolism of macromolecules are closely linked phenomena, since growth is necessarily the result of protein and nucleic acid synthesis. The fact that all the information that is characteristic of a living human being is stored in one fertilized cell, not only with regard to cell divisions involving billions of cells, and their structural and functional maturations but also to the time sequence of development of these characteristic events make us wonder with awe. There has been much progress in our scientific understanding of development, and much of this knowledge concerns the mechanism of protein synthesis in response to the transcription and translation of the genetic message. However, there are many gaps in knowledge about the exact way how and when the genetic message begins to be transcribed. The rate of net protein synthesis may be taken as an index of growth, since the increase in the mass of the cell may result from an increase in either the number of organelles within the cells or an increase in the soluble cytoplasm and both these types of increases involve synthesis of proteins. Protein synthesis maybe of two types: one is non-specific protein synthesis and the other is specific protein synthesis. Non-specific protein synthesis involves total protein synthesis during development, whereas specific protein synthesis involves the appearance or increase in the synthesis of different enzymes or special proteins characteristic of a particular kind of disease.

In the century and a half or more of the history of research into the proteins of nerve tissue, it is possible to distinguish two principal periods, the boundary between which is formed by the fifth decade of the twentieth century. In that decade radioactive methods, especially the use of amino acids labeled with radioactive isotopes, began to be used extensively for the study of the metabolic conversion of proteins in the nervous system.

The first period, which began in 1811 and which can be called the "pre-isotope" period, lasted about 140 years and ended with the discovery of the chemical topography of proteins in the tissues of the nervous system. For several decades in the nineteenth century, the attention of scientists was concentrated chiefly on the study of the lipid and carbohydrate composition of nerve tissue. As such, protein received far less attention. The main reason behind this was that the histochemical methods existing at that time and the biochemical methods that followed soon after were mainly for studying lipid and carbohydrate tissue components. Furthermore, for a long time the important role of proteins in the activity of the nervous system was not recognized. Studies revealed the complexity of the protein composition and the existence of protein complex with nucleic acids, lipids, carbohydrates and other substances in the nervous tissue. During the second or "isotope" period, although shorter in duration than the first, considerable progress has been made in studying the role of proteins in the functions of the nerve tissue. Because of the high sensitivity and high specificity of the isotopic indicator method, it is now possible to detect and to determine quantitatively, conversions of matter of such small magnitude in the living organism as were previously beyond the limit of methods earlier. Another unique feature of the isotope method is that the transformations of biologically important substances, especially proteins, on an extremely small scale can be detected in the intact organism without any significant disturbance of the functions of the organs of the experimental animal. In the case of dynamic equilibrium between the two processes, as is observed in the tissues of adult animals, the total protein content does not appreciably change, and for that reason,

the methods available previously could not provide information about the rates of protein synthesis or breakdown which can be obtained by the isotope technique. In developing animals, unlike the adult, there is a steady increase in the total content of proteins, nucleic acids, carbohydrates, lipids and other tissue components which can easily be determined by ordinary chemical methods. Even in this case, however, the use of labeled precursors has certain advantages, for, in conjunction with other modern methods it can be used to determine not only a change in the content of the proteins, carbohydrates and other substances in the tissue, but also the intensity and rate of the metabolic conversion of these substances, their individual fractions and individual components at all stages of ontogenic development. So by exploiting the radio isotope technique in the protein synthesis of developing organs, the differential rates of development in individual organs during fetal life, can be estimated.

All functions of the cell and all physiological phenomenon involve conversions of proteins. The elucidation of the biochemical basis of the various physiological functions of any cell, including the most highly organized and specialized nerve cell, is therefore impossible without the knowledge of the composition of proteins, their physiochemical properties and biological properties and the principles governing their conversions during cell function. Without minimizing the importance of other macro-molecular substances of the cell, it could be said that proteins do occupy a central part of biochemistry. The multiple nature of its functions are fascinating. All physiological functions and the biochemical processes on which they are based take place with the active participation of proteins.[21]. Some proteins perform enzymatic functions in the body, others have hormonal, protective, transport, structural and other functions [22–24].

Considerable progress has been made in the study of protein metabolism in animal tissues. The biosynthesis of tissue proteins takes place throughout development. In the developing organism this process provides chiefly for differentiation and growth of the tissues through the formation of their protein spectra, while in the later stages of ontongeny it functions chiefly for the renewal of the tissue proteins. Several evidences bearing on the morphogenetic changes in proteins in the course of differentiation of the various tissues during embryogenesis and the early stages of post-embryonic development have been summarized [25]. Regulation of protein composition, distribution and metabolism play a crucial role not only during the physiological functioning of the nervous system but also during cellular differentiation and during pathological changes. Before the widespread introduction of isotopes into biochemical research, age changes in protein metabolism were chiefly measured by determinations of the content of nitrogen and total proteins in organs, by changes in the qualitative composition and physiochemical properties of proteins and their complexes with other biologically important compounds and by the quantitative and the qualitative composition of the end products of dissemination. The first and fundamental indicator of these changes was the protein content in the brain at various stages of ontogeny. Evidences in relevant literature [26, 27] show that the protein content, calculated per weight of brain tissue, increases with age, although in old age its concentration falls [28]. During fetal life life there is a decrease in protein content (percent in relative population of solids) in the pig with the progress of gestation.

The process of protein synthesis has been extensively studied in the sea urchin [29]. In this primitive organism, protein synthesis is accelerated immediately after fertilization with an increased concentration of DNA, and the first signs of biochemical differentiation are apparent at the initiation of gastrulation [30]. Similar differentiation has also been observed at this stage of development in the amphibian embryo [31].

Substantial age and other functionally determined changes have been discovered in the chemical compositions [32], the structural organization [33] and the functional activity [34] of the cell organelles. These differences are concerned chiefly with the number, size and shape of the organelles [35] and also the distribution of protein [32], nucleic acids and free nucleotides and lipids [33].

Specific Protein Synthesis

Development is characterized by growth as well as by structural and functional differentiation. In the developing tissues and organs, specific enzyme patterns are expressed enabling the growing organisms to cope with the demands of fetal life after separation from the mother. Hence profound alterations in the enzymatic equipment of a growing and differentiating organ may be expected to occur.

Conclusion

The timing of the successive developmental processes is one of the most fundamental aspects of ontogenesis. The early phases of development depend primarily on the genetic program. However, the sequential gene expression is apparently under the control of metabolic influences as the dominant though perhaps not exclusive mechanism. For many decades, scientists have collected descriptive information about the morphological aspects of development, but the study of enzymatic development and differentiation and especially its regulation is comparatively recent. Enzymatic difference of any given tissue is the process whereby it acquires its characteristic quantitative enzyme pattern. This process involves both positive changes, the appearance or increase in synthesis of enzymes de novo, and negative changes, the diminution in the amount of other enzymes. The fundamental problem underlying this process is that of gene expression.

The carefully programmed normal formation of enzymes continues to a rather late stage in mammalian development, namely to the late fetal stage and early post natal period. It is only when enzymatic differentiation has been completed that any organ or organism reaches full physiological maturity and functions. The lack of complete enzymatic development forms the biochemical basis for the concept of 'functional immaturity'. Many clinical problems associated with infants of low birth weight, less matured infants and infants of diabetic mothers are those of functional immaturity or alterations in the normal course of enzymatic development. Knowledge and understanding of the normal process of enzymatic differentiation and its regulation, especially in the human fetus and neonate are of the greatest importance in the care of the newborn with problems of functional immaturity.

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Vaccination of the Unborn: A Perspective

2

Niranjan Bhattacharya and Sanjukta Bhattacharya

Introduction

Shortly after the Salk and Sabin polio vaccines had demonstrated the transformative benefits of childhood vaccination but long before the ill-informed controversy over the measles–mumps–rubella vaccine became a concern for refusal of vaccination, the Vaccination Assistance Act of 1962 established a U.S. vaccination program against polio, diphtheria, tetanus, and pertussis. With that effort launched and growing attention directed at imminent vaccination campaigns against influenza, measles, and rubella, a leadership group was formed by the US Government. That group, the Advisory Committee on Immunization Practices (ACIP), marks its 50th anniversary this year (2015) [1]. Vaccine refusal not only increases the individual's risk of disease but also increases the risk for the whole community. As a result of substantial gains in reducing vaccine-preventable diseases, the memory of several infectious diseases has faded from public consciousness and the risk–benefit calculus seems to have shifted in favor of the perceived risks of vaccination in some parents' minds [2].

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Theoretical Prospect/Potentialities of Vaccinating the Unborn

Infections are an important cause of morbidity and mortality worldwide and it is young infants who often suffer from a disproportionately great incidence of infection and subsequent mortality. Currently, vaccine strategies to prevent infections in infants generally direct initiation of immunization at around 2–4 months and protective levels of antibodies may not appear until 6–7 months of age [3]. This strategy fails to prevent important infections of the newborn in the first month. Experience with hepatitis B immunization of the newborn (both passive and active) shows that it can safely prevent neonatal infection and long term sequelae [4]. Tetanus in neonates has been dramatically reduced in many parts of the world in which pregnant women are vaccinated with tetanus toxoid [5].

Advantages and Disadvantages of Immunization

Strategy I (maternal immunization): Advantages: (a) a term baby has antibody at birth, and (b) there is active immunity in the mother. Disadvantages:

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(a) decreased passive antibody in premature infants, (b) passive immunity in the newborn baby wanes progressively within 6 months.

Strategy II (Neonatal Immunization): Advantages: (a) enhanced compliances at birth with mother's support; (b) the beginning of active immunity of the neonate. Disadvantages: (a) response to vaccination may be weak, and (b) development of protective immunity may take some more weeks.

Strategy III: Prospects of intra amniotic or intra fetal injection after 20 weeks; on the basis of the experimentation carried out by us (discussed in Chapter 4), there is abortion but no fetal death was reported in the growing fetus above 18 weeks.

Ours is possibly the first global experience with intrafetal vaccination attempts before 20 weeks of gestation. On the basis of our experiences we are strongly against vaccination of the unborn through the intra amniotic or intra fetal route, because of its potential adverse effects as seen in the histology of a non-aborted anencephaly fetus at 28 weeks and non-aborted fetus at 14 weeks leaving aside the problem of poor antibody response (depending on gestational maturity, time of exposure and the optimum dosage of antigen). The toxic/non specific reaction could be due to reduction in antibody formation and other forms of immuno-incoordination in the immature fetus.

While theoretically it may appear fascinating to vaccinate the grossly premature high risk newborn though the intra amniotic or intra fetal route and thus protect the premature newborn against possible infections, our practical experience goes strongly against such interventions at least not before 20 weeks.

Role of Antigenic Stimulation on Fetal Intra Uterine Growth: Implications of Increase in Liver Weight/Spleenic Weight/Thymic Weight and Rise of DNA in the Fetus Before the Process of Abortion

Antigenic (tetanus toxoid) stimulation through the intra amniotic route showed a definite increase in fetal liver weight, thymus weight, spleenic weight and also an increase in cell numbers when compared to the control (blank antigen) challenged fetus. There was also some increase in essential enzymes concerned with glutamine synthesis of the human fetal liver glutamyl dehydrogenase and gamma glutamyl transferase. Seeing such changes within the wider physiological content, attention has been increasingly drawn to the interactions between the immunological and the neuroendocrine systems. Immunological cells have the receptors which enable them to receive signals from a wide range of hormones, corticosteroids, insulin, growth hormones, testosterone, estradiol, B adrenergic agents, acetylcholine, endorphin and encephalins, but by and large, glucocorticoids and androgens depress immune response whereas estrogen, growth hormone, thyroxine and insulin do the opposite [6]. These hormones work through different cytokines/growth factors and their binding protein or proteases. Hence the precise mechanism of how the antigenic stress at a critical period of growth in vitro possibly disrupts the fine immune-neuroendocrine coordination of the receptor growth factor cytokine mediated action at the cellular level, would be an interesting field of research for future researchers in this area.

Theoretical Problem of Graft versus Host Reaction and Autoimmunity in the Aborted and Non Aborted Fetus

The rise in spleenic weight and liver weight along with the necrosis of the liver, denudation of intestinal epithelium and skin lesion constitute the cardinal features of GVH disease [7]; hence its presence strongly suggests the possibility of graft versus host type of injury effect with antigenic challenge through the intra amniotic route due to a disruption in the thymic schooling of the T lymphocyte during the human fetal ontogeny of the immune system. Spleenic rise in weight from 12 weeks onwards and pre-spleenic, i.e., before 11 weeks, liver weight rise, which continued even after the appearance of the spleen, up to 20 weeks of the study of a human gestation with intra amniotic antigenic challenge as well as

study of the histology of the skin/liver, suggests the possibility of graft vs host type of reaction effect. This hypothesis regarding graft vs host reaction may not hold true for the early weeks of gestation of a fetus, that is, before 16 weeks, because antigens cause direct stimulation of the fetal system, resulting in congestion, hemorrhage, varying degrees of loss of architecture in the early weeks and mononuclear invasion and thrombosis in all the organs and in the placenta with varying degrees of villitis in later weeks (17 weeks or more). We believe that the early acute reactions in the embryo in the pre-immune status of the fetus (16 weeks) are hitherto unnamed inflammations or perhaps even an autoimmune reaction in case there is participation of the premature immune system, while reactions in the 17 or more weeks fetus may have a component of graft vs host reaction as may be perceived from the placenta with villitis, thrombosis, mononuclear invasion etc. However, further specialized immunological study, which will differentiate the fetal leucocyte from the maternal leucocyte, can confirm or reject our hypothesis that antigenic intra amniotic stimulation during the early weeks of gestation can cause an autoimmune type of acute reaction due to poor schooling at the thymus. The fetal lymphocytes would be able to distinguish properly or at least partially, its own property (antigen) from the foreign property (antigen) and the war would be at the fetomaternal border (in the placenta). Conscription of the immature fetal leucocytes due to antigenic stress or war like situations in the fetus, can provoke the much more powerful (superpower) maternal immune systems' sanctions which can lead to premature birth (independent immature systems statehood for the fetus) or death and abortion depending on the gestational maturity of the fetus.

Advantage of Hypoimmune Fetal System in Biomedical Research

What have we learned from our study of antigenic stimulation of the growing human fetus in the utero for the last 40 years? We have learned that the growing human fetus (8–20 weeks) in utero can react to an antigenic challenge; for humoral immunity (tetanus toxoid) and cellular immunity (BCG). However, in case of tetanus toxoid stimulation the effect widely varies, as there is wide variation in the induction-abortion interval. Why is this so? Is it the lower expression of receptors, or neoantigens in the pre-immune or the hypoimmune fetal system which make it resistant to antigenic stimulation? The answer is possibly in the affirmative. This could explain the delayed response even with a very high dosage of antigenic stimulation (2–4 cc tetanus toxoid) for a tiny fetus weighing 8–150 g in the uterus, when the adult dosage is 1 cc.

This hypoimmune fetal tissue has an edge over the adult tissue in situations requiring transplant of fetal tissue in the adult system. For example, in case of stem cell transplantation (umbilical cord blood stem cell) in adults or fetal thymus transplantation (unpublished observation), there is survival of the fetal tissue without any graft versus host reaction in the adult system [8–11].

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3 Understanding the Growth of the Fetus in Utero from the Immunologists' Angle

Niranjan Bhattacharya and Phillip G. Stubblefield

Development and Growth of the Human Immune System in Utero

Developmental immunology can be defined as the study of how adaptive host defense blood cells within an individual sequentially respond to repetitive environmental challenges in a way that promotes the health and survival of an individual. According to classical principles an individual becomes immune or protected from re-infection in response to an antigenic encounter during an initial infection. Mature immunological competence is ultimately achieved through cumulative adaptive changes stimulated by exposure to a large repertoire of foreign antigenic material. Since the in utero fetal environment is sequestered from frequent encounters with micro-organisms, the host defense system of the human newborn is inexperienced. This partially accounts for why the human newborn is vulnerable to human microbial attacks during the first 6 weeks of life. Furthermore although many components of the immune system of the fetus are present early in gestation some are immature and do not become fully functional (compared with the activity of immune defenses of adult subjects) until sometime after birth. Despite these limitations fetal host defense systems are capable of active engagement and an immune response does occur when the fetus is infected or immunologically challenged in utero.

The cells involved in the human immune system are derived from the stem cells originating in the yolk sac. In the human system, fetal liver and bone marrow takes the responsibility of the yolk sac at 5 weeks gestation. Natural Killer (NK) cells and T and B lymphocyte precursors are detectable in the fetal liver at 6 weeks and 7–8 weeks gestation. The fetal thymus is colonized by T cell precursors at 8–9 weeks and pre B cells are found in the bone marrow at 13 weeks, as evidenced due to the attachment of cluster of differentiation (CD) molecules in each of the sub types. Mature T and B lymphocytes are detectable by the onset of second trimester pregnancy. Some complement components can be detected from 6 to 14 weeks gestation although at a much lower level than in the adult.

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Natural Immunity (Cellular Component of Macrophage Monocyte Natural Killer Cell System)

In the fetus polymorphonuclear (PMN) leucocytes are first identified at the fetal yolk sac stage of hematopoiesis [1]. Mature PMN's are not identifiable in the fetal liver or bone marrow until approximately 14 weeks of gestation [2]. By 22–23 weeks of gestation the circulatory PMN count has increased but still it is only 2 % of the circulatory PMN concentration measured in the cord blood of term gestational newborns. This low PMN count is compensated with high hematopoietic progenitor cells. This may be due to shifting of stem cells from the liver to the bone marrow.

Before the liver becomes the major site of hematopoietic activity, macrophages constitute 70 % of the blood cells present in the liver. Circulating monocytes do not appear in the fetal blood before 20 weeks of gestation. However, at 30 weeks gestation, monocyte concentration reaches 3–7 % of the circulating formed blood cells. As a whole, the influx of mononuclear phagocytes to the site of inflammation is delayed and attenuated in the new born. Similarly, in the case of NK cells whose number is normal in the neonatal period, the surface membrane expression of certain antigens is altered in comparison to adult NK cells. This may explain the diminished cytotoxicity in vitro and vulnerability of the newborn to viral and parasitic infection.

Natural Immunity (Humoral Component)

Opsonin activity is reduced in the new born at term and more so in the prematurely born at test concentration of plasma and serum above 10 %. This deficiency may be in part related to the lower complement or immunoglobulin (IgM and IgG) concentration in the new born [3].

Complement proteins are synthesized early in gestation [4]. Synthesis of C4, C2, C3 and C5 can be confirmed between 8 and 14 weeks of gestation. Evidence derived by several methods confirms that there is no transplacental passage of complement components. Components of the alternate pathway activity are more deficient in concentration than classical pathway activity in both term and pre-term infants.

Fibronectins are a class of multifunctional high molecular weight glycoproteins that serve to facilitate cell to cell to sub stratum adhesion and thus play an important role in directing cell migration, proliferation and differentiation. Thus fibronectins are essential for certain aspects of embryological development of the fetus and for hemostasis, hematopoiesis, inflammation and wound healing. In many pathological conditions like sepsis, fibrosis, etc the normal structure, physiology and functions of fibronectin are altered in a way that contributes to the underlying tissue or organ dysfunction. Circulatory plasma fibronection concentrations are reduced in fetal cord blood and in the term infant [5]. Plasma fibronectins are further reduced in respiratory distress syndrome, birth depression, sepsis, and intra uterine growth retardation. Fibronectin biosynthesis by macrophages in vitro is decreased in the neonatal period [6]. Fibronectin improves leucocyte function in vitro and promotes neutrophilic adhesion migration, chemotaxis and also assists killing of opsonized bacteria, yeasts etc.

Other Humoral Factors of Natural Immunity

C reactive protein (CRP) is synthesized by the fetus and the newborn and is one of the most important acute phase proteins. It helps to activate the classical complement pathway by beading with the C1q. CRP binding with the bacteria helps in the opsonization and rapid clearance of the bacteria through the neutrophil, monocyte or macrophage phagocytosis.

Lactoferrin is a positively charged iron binding glycoprotein present in the granules of the neutrophils. This helps in neutrophilic reactive oxygen intermediate production (OH), chemotaxis, endothelial adhesion, aggregation etc. Neonatal cord blood neutrophils are profoundly deficient in lactoferrin [7].

Acquired Immunity-Cell Mediated and Antibody Mediated Responses of T Lymphocytes

In the human, the thymus develops embryologically as an outgrowth from the third and fourth pharangeal pouches between the sixth and

seventh week of gestation. Lymphocytes destined to become T cells appear among epithelial cells during the ninth and tenth weeks [8]. However it is not until the tenth week of gestation that the cortex and the medulla of the thymus region begin to demarcate and not until the 12th weeks of gestation that Hassall's corpuscles appear. The undifferentiated cells that first enter the thymus at the seventh week of gestation do not possess either CD4 or CD8 antigen but do express the T cell markers [9]. Within the thymus, maturation of the T cells is accompanied by the sequential appearance of surface phenotype markers CD7 followed by CD1, CD2, CD5 and somewhat later, CD3. As gestation progresses most cells leaving the thymus express either the CD4 or CD8 surface antigen. Those cells that lack both the antigens retain the stem cell function and possess a receptor (CD25 Tac antigen) for IL2 which plays an essential role in promoting simultaneous appearance of CD4 or CD 8 antigens. At this stage, the transcribed T cell alpha chain precedes that of the Beta chain. At the 12th week of the intra-uterine of life in the fetus, CD3 positive cells can be identified at the peripheral blood, which increase progressively with the progress of gestation and represent more than 50 % of T lymphocytes by the 22nd week of gestation. These CD3 positive cells also express either the CD4 or the CD8 antigen. By the 13th week of gestation CD3 positive cells appear in the fetal liver or spleen and by the end of the second trimester represent more than 50 % of the T lymphocytes in those organs. The T cell helper/suppressor ratio (CD4/CD8 ratio) in the cord blood is approximately 1.7:1 (versus the adult ratio of 2:1). In the peripheral blood 20 % of the T cells express thymocyte antigens compared with fewer than 1 % expression in the adult [10].

Lymphokines Production and Role in Cell Mediated Immunity

Functional responses involving the T lymphocytes and T cell derived lymphokines can be demonstrated relatively early in gestation. By the 12th week of gestation, lymphocytes obtained from the human thymus respond both to mitogens and to foreign histocompatiblity antigens in mixed lymphocyte culture [11]. In addition fetal cells stimulated with allo-antigens exhibit normal antigen specific cytotoxicity. In contrast, the phenotype appearance and proportion of circulatory cells is diminished and the production of some lymphokines are reduced in the neonate. The most significant defect appears to be a deficiency of memory T cells, because expansion of the memory T cell production is dependent on the exposure to foreign antigens. This T cell inexperience may be partially responsible for the diminished production of gamma interferon in the neonate [12].

B lymphocyte and Antibody Production: B cell maturation occurs in two stages. In the first stage undifferentiated stem cells mature into cells identifiable as B lymphocytes. This is an antigen independent phase that takes place in the fetal liver and the bone marrow in humans [13].

The second stage of lymphoid differentiation is antigen dependent and during this phase B lymphocytes are transformed into plasma cells. The first recognizable cell in the B cell lineage is the pre B cell. This cell can be detected in the fetal liver in the seventh to eighth week of gestation. The presence of heavy chain IgM [14] could be seen at this stage. As gestation progresses, pre-B cell can be detected in the fetal bone marrow. It is at the pre-B cell stage of development that clonal diversity is generated. Intact immunoglobulin genes are formed by the re-arrangement of gene segments comprising each heavy and light chain family. Pre-B cells also give rise to immature B lymphocyte which expresses only surface IgM and complement receptors [15]. These cells could be detected in the fetal liver at 8–9 weeks of gestation. However, clonal anergy could be noticed in case of exposure to self-antigen. Cells that express other immunoglobulin IgA or IgG can be demonstrated by the 12th week of gestation. At a somewhat later state (the mature B cell stage), cells express membrane bound IgG or IgA in association with the membrane IgM or IgD. By the 12th week of gestation, the normal fetus has levels of circulating B lymphocytes that are equal or higher than the adult. Fetal B lymphocytes can be demonstrated in highest proportion in the spleen (30 %), blood (35 %) and lymph nodes (13 %).

Antibody Production in the Fetus and Neonates

The fetus acquires the ability to produce serum immunoglobulins early in gestations [16]. In vitro studies have demonstrated the ability of fetal cells to produce antibody (IgM) by the eighth week gestation. IgG synthesis appears slightly later and IgA synthesis begins at about 30 weeks of gestation. However due to the sterile environment of the uterus the inability of the fetus to respond to certain kinds of antibody, T cell suppression and B cell differentiation, results in a very little antibody level before the time of birth [17]. At the time of birth most of the circulating antibodies in the fetal system are IgG antibodies passed through the placenta. Low level of fetal IgM (less than 10 % of the adult level) are demonstrable at term gestation and reach the adult level by 1–2 years of age. Adult level of IgG is attained at 4–6 years and adult IgA level is attained at puberty.

Placental Transfer of Antibody

Although B lymphocytes are present in the fetus, by the end of the first trimester there is very little active fetal immunoglobulin production. Without exposure to an antigen in the sterile environment of growth and differentiation of the fetal system serum immunoglobulin, levels are extremely low until 20–22 weeks of gestation, at which time an accelerated active transport of IgG takes place across the placenta [18]. Only the maternal IgG is transported due to the presence of specific placental receptors for heavy chain IgG molecules. This active transport of IgG causes a rise of IgG concentration in the fetus 5–10 % higher than the mother. Elevated levels of IgM or IgA in the cord blood demonstrates that the infant has been exposed to an antigen in utero and has synthesized antibody itself. Congenital infection with syphilis and rubella characteristically produces elevation of the cord blood IgM concentration. Elevated levels of both IgM and IgA also maybe found if there is transplacental materno-fetal hemorrhage.

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4 Intraamniotic Antigen and Disruption of Human Fetal Growth: A Study from 1978–2002 with Subsequent Follow Up

Niranjan Bhattacharya

The search for a safe, effective, mid-trimester abortifacent (1978–2002) in which a group of researchers were engaged in Calcutta, revealed many hitherto unknown secrets about the growth and development of the human fetus up to 20 weeks. A major finding was that while expulsion by intra amniotic tetanus toxoid or auto absorption in case of BCG is the effect, the cause is the disequilibrium of the coordination of the growth and maturation of the developing fetus. That the fetus, even at a very early stage of growth, can react to an antigen, even in a sterile environment leading to embryopathic changes at the teratogen phase (up to 9 weeks) or the pre-immune phase (10–16 weeks), in a non-specific manner resembling an acute non-specific infection, or the hypo-immune phase (from 17 weeks onwards), is a new finding. These finding may have serious implications for future medical research. The major thrust areas in this context are discussed below.

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Intra Amniotic Antigens as Abortifaecients

This article will showcase our experience of 688 cases of abortion after single intra amniotic injection of 2 cc tetanus toxoid (Table 4.1). It was observed that if the period of gestation is randomized the overall success rate of abortion after a single injection is 92.80 % in the present study. When the cut off period was 7 days after intra amniotic 2 cc injection, the abortion rate was 52.8 % (363 cases) which cumulatively became 72.4 % (498 cases) if the cut off period was extended for another week. On the 21st day cut off schedule, the rate was 86.2 % of abortion (593 cases), which figure eventually shot up to 92.8 % (638 cases) when the cut off period was 30 days. In sum, the experience showed that with 2 cc single intra amniotic injection of tetanus toxoid, there was 94.74 % abortion in the 8–11 weeks group and 94.05 % in the 12–15 weeks group, if the cut off period was 1 month. However, the induction-abortion interval varied widely. With progression of gestation 16–19 weeks or more, there was a gradual increase in the induction-abortion interval as is apparent from the mean induction-abortion interval. The success rate (if there is a cut off period of 1 month) of a single dose of intra amniotic injection of 2 cc schedule progressively comes down from 94.74 % in the 8–11 weeks group to 88.61 % in the 16–19 weeks

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Table 4.1 Intra amniotic tetanus toxoid single injection 2 cc series cut off period for success rate in 30 days

No. of cases (in %)	Weeks of gestation	Type of antigen used	Induction abortion interval range (mean ± SD)	Success rate (in %)	Condition of abortus (living)	Dead
19	8–11 weeks	Tetanus toxoid 2 cc single intra-amniotic injection schedule	48 hours–11 days hour mean with SD = 96 h ± 24.6 h	94.74	–	19
319	12–15 weeks	Tetanus toxoid 2 cc single intra-amniotic injection schedule	56 h–13 days 4 h mean with SD = 118 ± 32.4 h	94.05	–	319
316	16–19 weeks	Tetanus toxoid 2 cc single intra-amniotic injection schedule	76 h–26 days 4 h mean with SD = 14 h ± 48.6 h	88.61	36 fetuses all above 16 weeks	280 dead fetuses
34	20 weeks and above	Tetanus toxoid 2 cc single intra-amniotic injection schedule	116 h–28 days 4 h mean with SD = 186 ± 36.4 h	73.67	All fetuses living	–

group and eventually to 73.67 % in the 20 weeks or more group. But after randomization of gestation from 8 to 20 weeks the overall success rate, after single intra amniotic tetanus toxoid 2 cc injection, was 92.80 % in the present study.

It appears from Table 4.2 that with a multiple injection schedule of 2 cc intra amniotic tetanus toxoid weekly among the non-aborters there were two cases of failure on the 21st day, i.e., 8.33 % failure (even with multiple injection). It may be mentioned here that in the multiple injection schedule, certain associated problems can be found. Apart from inconvenience to the patients, there is a distinct possibility of fetomaternal exchange. If the fetomaternal exchange is 1 % with the USG amniocentesis protocol, it eventually becomes 3 % with the third prick/attempt on the non-aborters. In case of clinically guided amniocentesis (on the side of the fetal limbs or behind the fetal back in a bigger fetus) the cumulative percentage of fetomaternal exchange would be around 10 % or even slightly more. Under these circumstances, ascertaining the specific effects of tetanus toxoid would be difficult, leaving aside the problem of immune paralysis, because the effective immune response of an antigen requires a synchronization of a number of different physiological and immunological events. The immune system is controlled and regulated by the genetic background. The specific responses of all antigens are determined by the immune response (Ir) gene and the immune suppressor gene (Is) with the development of its peripheral systems and sub systems including control of protein synthesis, located at the major histocompatibilty complex. Another problem in the adult or mature system is the nutritional status of the individual. Further, we are not certain about the receptor expression in the different afferent immune arch of the growing fetus. Seen from a phylogenetic angle, the embryonal and fetal growth in vitro shows a wide species variation so far as the afferent immune arch is concerned. Summing up, it appears that to generate an ideal immune response, an antigen (vaccine) should have a few general requirements like the (a) activation of antigen presenting cells to initiate the antigen processing, (b) the activation of both B and T cells to provide memory cells, (c) the generation of response to a variety of epitopes, (d) the persistence of the antigen so that the immune system remains stimulated for a long time. And most importantly, (e) safety.

All ante-natal mothers globally receive routine intra-muscular vaccination of tetanus toxoid. In this study, the objective was to observe the impact of tetanus toxoid which is an antigen, when it is injected intra-amniotically or

Table 4.2 The effect of intra amniotic multiple 2 cc injection 7 days apart on the growing human fetus up to 20 weeks

Age	No. of cases	Weeks of gestation	1st injection to wait till day 1–7 Success within 7 days	1st injection to wait till day 1–7 Failure	2nd injection to wait till day 8–14 Success within 14 days	2nd injection to wait till day 8–14 Failure	3rd injection to wait till day 15–21 Success within 21 days	3rd injection to wait till day 15–21 Failure	Comments
18–30 mean age in years with SD 28.2 ± 1.4	24	Range 8–20 weeks gestation mean with SD = 16.6 ± 1.4 week	11 (45.8 %)	13 patients	11 + 9 = 19 79.1 % cumulative success rate after 2nd injection within 14 days	5 patients	11 + 8 + 3 = 22 91.6 % cumulative success rate after 3rd injection within 21 days	2 patients	

intra-fetally in an immature or a growing immune system on the growth, maturation and development of the fetus. It may be mentioned here that medical termination of pregnancy is legally permissible in India up to 20 weeks for the purpose of family planning. A number of government hospitals provide this service free of cost. As such, many mothers come to these hospitals for termination of pregnancy, which is done on a regular basis along with suggestions of other ways of prevention of pregnancy in future. Standardized procedures are followed like prostaglandin injection/vaginal application/oral application apart from hypertonic solutions like urea, manitol, saline etc. If any problems occur or there is failure of abortion or anaphylactoid reaction, patients are given the option of intra-amniotic antigen experimentation. Tetanus toxoid is the commonly used antigen because of its safety profile, easy availability and low cost.

Clostridium tetani is the causative agent of tetanus which is one of the important species of the genus clostridium, which comprises of spore forming gram positive anaerobic bacilli. Two products liberated by C. tetani are the classical neurotoxin (tetanospasmin) and haemolysin (tetanolysin). The haemolysin is heat liable and inactivated by oxygen. All the symptoms in tetanus are attributable to an extremely toxic neurotoxin. Tetanus toxin is produced in vitro in amounts up to 5–10 % of the bacterial weight. The purified neurotoxin is a simple protein containing no carbohydrate and with a molecular weight of 150,000 daltons. The toxin exists in two stages: the toxin monomer and a dimer of about twice the molecular weight. The dimer is non-toxic but antigenic. Treatment of this toxin with formaldehyde results in polymerization of toxin and results in the formation of toxoid which is non-toxic but antigenic. In the detoxification procedure, a standard hypertoxigenic strain (Harvard strain) of C.tetani is used. In case of an adult/baby, the number of Lf of toxoid should not exceed 25 Lf for primary immunization. The immunization of all women in the child bearing age in tetanus endemic areas has been recommended by the World Health Organization (WHO). There is no specific time period of pregnancy when women should be immunized against tetanus. In those countries where the risk of tetanus neonatorum is low, usually the immunization is deferred during pregnancy. It has been seen that immunization during the fifth and eighth months of pregnancy results in the formation of antibodies in the infant and also enhances the response of these infants to subsequent immunization. This phenomenon has been termed as transplacental immunization.

Though extremely rare, certain adverse effects of tetanus vaccination have been reported viz., local reactions like swelling, redness and pain up to 10 days at the site of injection; then there are systemic reactions like pyrexia, myalgia, malaise, acute anaphylaxis, peripheral neuropathy, elevated IGE level and elevated Anti A and Anti B antibodies. Very occasionally a reaction has been seen in individuals who had high levels of circulatory anti toxin and in whom bolstering was attempted. Tetanus toxoid has been reported to increase Anti A and Anti B antibodies owing to the traces of blood group antigen in the vaccine. Immunization of patients (pregnant) with the aim of preventing neonatal infection may therefore increase the risk of hemolytic disease of the newborn [1].

With this backdrop of information in mind, let us now explore our results. The problem is what should be the ideal dosage for embryonal and fetal immunization through the intra amniotic route. As we do not have a set of rules to follow and there is prevailing confusion over the degree of fetal and amniotic fluid exchange of secretions and excretions and the subsequent dilutions and exchanges with the maternal compartment, the problem is how much antigen injected through the intra amniotic route can cause stimulation of the fetal system (a) non-specifically and (b) specifically, with the progressive maturation and the gradual development of the fetal immune system. This is a most important gap in the existing state of knowledge.

Tables 4.3, 4.4, 4.5, 4.6, and 4.7 demonstrate the effects of intra-amniotic tetanus toxoid injection, varying from ½ to 4 cc (single dose).

Table 4.3 Intra amniotic tetanus toxoid with changes in dosage (single ½cc age parity injection) schedule

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
28.6 ± 4.4	2.8 ± 0.6	20 patients	9–19 weeks. Mean gestation with SD 13.8 ± 1.2	½cc tetanus toxoid (single injection)	48 h–13 days 2 h	12 patients did not abort on the 7th day, 6 patients did not abort on the 14th day

Table 4.4 Intra amniotic tetanus toxoid 1 cc series (single injection)

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
32.6 ± 1.4	3.4 ± 1.2	14 patients	8–19 weeks. Mean with SD 14.4 ± 2.4	1 cc tetanus toxoid (single injection)	72 h–11 days 14 h	10 patients did not abort on the 7th day, 4 patients did not abort on the 14th day

Table 4.5 The effect of intra amniotic 1.5 cc tetanus toxoid single dose on the growing human fetus

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion Interval range	Comments cut off period 14th day
33.8 ± 1.8	2.8 ± 0.6	16 patients	9–19 weeks. Mean with SD 12.6 ± 1.2	1.5 cc tetanus toxoid (single injection)	68 h–11 days 12 h	6 patients did not abort on the 7th day, 4 patients did not abort on the 14th day

Table 4.6 The effect of 3 cc single intra amniotic injection of tetanus toxoid on the growing human fetus in the below table

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
34.2 ± 2.8	3.6 ± 1.2	10	8–18 weeks. Mean with SD 14.6 ± 2.8 weeks	3 cc tetanus toxoid (single) injection intra-amniotically	112 h–12 days 16 h	6 patients did not abort on the 7th day, 3 patients did not abort on the 14th day

If we analyze the gestation randomized results of Tables 4.3, 4.4, 4.5, 4.6, and 4.7, we find that the abortion failure rate on the seventh day with ½cc tetanus toxoid is 60 % (n = 20); with 1 cc the failure rate on the seventh day is 71.42 % (n = 14) and with 1 ½cc tetanus toxoid, the failure rate on the seventh day is 37.5 % (n = 16) and with 2 cc tetanus toxoid the failure on the seventh day is 47.2 %, and again with 3 cc tetanus toxoid dosage the failure rate is 60 % (n = 10) on the seventh

Table 4.7 The effect of single intra-amniotic 4 cc tetanus toxoid on the growing human fetus as shown in the table below

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
29.6 ± 2.8	2.8 ± 0.6	12 cases	8–19 weeks. Mean with SD 14.6 ± 2.4	4 cc single injection intra-amniotically	110 h–13 days 12 h	8 patients did not abort on the 7th day, 4 patients did not abort on the 14th day

day; again with 4 cc tetanus toxoid dosage the failure rate on the seventh day is 66.6 % (n = 12). Now if we analyze the cumulative failure on the 14th day we will get the result of failure of abortion with 1/2 cc of tetanus toxoid on the 14th day single injection to be 30 % (Table 4.3). With 1 cc tetanus toxoid intra amniotic injection the cumulative failure rate on the 14th day is 42.85 %. With 1 1/2 cc of tetanus toxoid intra amniotic regime of single injection the cumulative failure on the 14th day is 25 %. However, with 2 cc of tetanus toxoid injection the cumulative failure rate is 27.6 %. With 3 cc intra amniotic tetanus toxoid regime, the cumulative failure of abortion on the 14th day is 30 % and lastly, in the 4 cc single intra amniotic antigen injection cases, the cumulative failure of abortion on the 14th day is 33.3 %. In all such cases from Tables 4.3, 4.4, 4.5, 4.6, and 4.7, the gestation period has been randomized (8.9–19 weeks from LMP). If the results are compared from ½ to 4 cc of single dose of tetanus toxoid with the multiple injection schedule of Table 4.2, it appears that there is a marginal superiority (n = 24) with multiple injection of Table 4.2 where cumulative success rate is 79.1 % (n = 24) on the 14th day as against 72.4 % with 2 cc single injection. However, this inference is too simplistic, because then we have to ignore the cumulative possibility of fetomaternal hemorrhage as a sequel to each injection and the immaturity of the memory cells of the fetus.

During discussions with Prof. Arnold Klopper of the Royal College of Gynaecologists and Obstreticians at the time of his visit to our laboratory on 18th January 1980, Prof. Klopper suggested that the unpredictability of fetal stimulation can be overcome with intra-fetal injection under ultrasound control [4]. As a matter of fact this event appears to be the first attempted vaccination of the human fetus in utero. Here, too, there are certain technical problems, e.g., fetal movement, and we presume there may be even some regurgitation of the antigen to the amniotic cavity depending on the site of injection, needle bore, fetal movements etc. Similarly though we always tried to target the fetal back muscles for intra-fetal injection, inadvertent injury to fetal vital structures like liver, lung and surrounding structures cannot be ignored.

The following Tables depict the results of dosage variation in the injection schedule (Table 4.8).

Though the series is small (n = 12) with single injection schedule, 50 % of those injected aborted within the seventh day and cumulative 83.33 % abortions were noted on the 14th day, which is possibly the highest performance, even better than with the 2 cc schedule 72.4 % success and multiple injection schedule 79.1 % success rate on the 14th day. However, here too, interpretation on the basis of simple statistics would be too simplistic because we have to ignore then the effect of injury to the fetal vital structure with the antigen injection process and immaturity of the fetal immune system.

In one experiment, we wanted to see whether the addition of a fetotoxic or immunomodulatory substance like Vitamin A in the dosage of 300,000 IU if injected with tetanus toxoid can change the scenario. Here again we found that in 70 % cases there was failure of abortion on the seventh day and a cumulative 40 % abortion failure was noted on the 14th day (Table 4.9).

Table 4.8 The effect of direct 2 cc intra-fetal injection under ultrasound guidance on the growing human fetus up to 18 weeks

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
26.64 ± 2.6	3.2 ± 1.2	12 patients.	12–18 weeks. Mean gestation with SD 12.6 ± 1.4	Single intra fetal injection of 2 cc under USG'	68 h–11 days 14 h	6 patients did not abort on the 14th day, 2 patients did not abort on the 14th day

Table 4.9 The effect of an immunomodulation and fetotoxic substance, vitamin A and 2 cc of tetanus toxoid on the growing human fetus up to 18 weeks

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
24.6 ± 2.6	2.6 ± 0.4	10	8–18 weeks. Mean with SD 14.6 ± 2.4	Single intra amniotic injection of 2 cc tetanus toxoid and 300,000 IU of Vitamin A	48 h–13 days 12 h	4 patients did not abort on the 14th day, 7 patients did not abort on the 7th day

Intra-amniotic tetanus toxoid 2 cc + 300,000 IU of Vitamin A

Table 4.10 Intra amniotic tetanus toxoid 2 cc and oral levamisole (150 mg daily) to mother for 7 days uninterrupted

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
28.6 ± 1.4	2.9 ± 1.9	12	8–18 weeks mean with SD 13.8 ± 0.8	Tetanus toxoid 2 cc with levamisole orally to mother	49 h–11 days 14 h.	7 patients did not abort on 7th day. 3 patients did not abort on the 14th day

Table 4.11 The effect of maternal non-immunization and subsequent intra amniotic instillation of 2 cc tetanus toxoid on the growing human fetus up to 20 weeks

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
34.2 ± 2.8	3.6 ± 1.2	16	8–19 weeks mean with SD 14.2 ± 1.4 weeks	2 ml of single fixed dose of intra-amniotic injection	51 h–8 days 12 h	6 patients did not abort on 7th day. 4 patients did not abort within 14th day

Intra amniotic tetanus toxoid without maternal immunization of tetanus toxoid

In order to understand whether maternal T cell stimulation or modulation of T cell function pharmacologically can make the induction of abortion interval more predictable, we utilized oral levamisole (150 mg OD × 7 days). Here 58.33 % cases failed to abort on the seventh day and 25 % cases failed to abort on the 14th day (Table 4.10).

Table 4.12 Intra amniotic tetanus toxoid 2 cc along with oral cimetidine 400 mg BD to mother for 7 days uninterrupted

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
33.4 ± 1.4	3.6 ± 1.2	14	8–19 weeks mean gestation with SD 13.6 ± 1.4 weeks	2 cc of intra amniotic tetanus toxoid and oral cimetidine 400 mg BID to mother	52 h–13 days 12 h	8 patients did not abort on 7th day. 6 patients did not abort within 14th day

Table 4.13 The effect of intra amniotic 1 cc glutamate BCG on the growing human fetus up to 15 weeks

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments cut off period 14th day
33.3 ± 1.4	3.4 ± 1.2	26	11–15 weeks mean gestation with SD 13.2 ± 3 weeks	1 cc of glutamate BCG	No abortion varying degree of dissolution vis-à-vis auto absorption of the fetus on the 14th day	Mothers who were Mantoux test negative were enrolled. Mantoux test positive and ELISA Tb IgA, IgG and IgM (+) cases were discarded from study design

The Table 4.11 shows mothers who were not immunized with tetanus toxoid. We wanted to see the role of maternal immunization on the induction-abortion interval of the growing human fetus before 20 weeks, when challenged by tetanus toxoid through the intra-amniotic route. Here, we find 62.5 % cases aborted within 7 days and 75 % cases of abortion within 14 days. Excepting for these 15 subjects in the above Table, all other mothers were properly immunized with tetanus toxoid.

In Table 4.12, the results of mothers who were adviced to take cimetidine (H2 receptor antagonist) 400 mg BD dosage along with intra amniotic tetanus toxoid 2 cc injected are shown. Here also we found that eight cases did not abort on the seventh day (57.14%) and six cases did not abort on the 14th day (42.85 % failure).

In Table 4.13, we have shown our experience with intra amniotic instillation of BCG. All commercial BCG vaccinations owe their parentage to the culture developed by Calmette and Guerin about 60 years ago. Subsequently daughter strains were named differently such as Montreal, Connaught, Glaxo and so on. The standard dose of BCG vaccine is 0.1 mg in 0.1 ml volume injected intradermally. The protective efficacy of BCG vaccination has been the subject of considerable controversy. There have been nine major internationally controlled trials with BCG vaccination in various parts of the world during the past 60 years. While highly significant protection (70–80%) has been obtained in several trials conducted in Europe, Canada and the USA this has been offset by a report of an almost total lack of protection (0–37 %) in Puerto Rico and Chengleput (South India) [1]. There are many explanations of which the most plausible is that the immune reactivity of the population had been conditioned by prior exposure to the mycobacterium in the environment. Contra indication for inclusion in the above Table for BCG trial is Mantoux test positivity. (Later, as per Ethical Committee suggestions, ELISA Tb IgG, IgM,

Table 4.14 The effect of intra amniotic double antigen challenge on the growing human fetus up to 20 weeks

Mean age in years with SD	Mean parity with SD	No. of Cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion Interval range	Comments
25 ± 2.4 years	2.8 ± 6	14	10 to 16 weeks. Mean gestation with SD 13.4 ± 1.4	0.5 cc of dyphteria and 0.5 cc antigen commonly known as double antigen	88 h–12 days 12 h	6 patients aborted within 7 days. 10 patients cumulatively aborted within 14 days

IgA studies were made mandatory before inclusion in this trial).

As we have stated earlier, due to the prevailing unpredictability of the actual amount of antigens entering the fetal circulation due to several factors, we used 1 cc of glutamate BCGH intra amniotically in all the cases (Table 4.13) irrespective of gestational period, i.e., 11–15 weeks in this study. To our utter surprise there was no abortion. However there was progressive diminution of the size of the uterus. When we did hysterotomy on the 14th day after the injection, the uterus was found to be practically free of the conceptus. We repeated the experiment again and again, but the result remained the same on the 14th day of the instillation of BCG. Twenty-six such cases were experimented with and the gestation period varied from 11 to 15 weeks. However, when analyzing the data, the question arises why pregnancies above 15 weeks were not included. The answer to this question is the restriction suddenly imposed by the Institutional Ethical Committee on BCG experimentation on the plea that persistence of placenta or any other fetal tissue in the maternal system may have the possibility of inducing hydatidiform mole or choriocarcinomatous changes to mothers in the long run.

In the animal system, auto absorption is seen in the conceptus frequently, especially in cases of multiple pregnancies. In human pregnancy we have frequently noted a vanishing twin or a part of the gestational triplet or quadruplet. There, placental vascular factors have been primarily or secondarily attributed as the cause. However, there are published reports of successful pregnancy after selective termination of multiple pregnancy with KCL or subsequent normal pregnancy after treatment of ectopic pregnancy with methotrexate. Hence, there should not be undue fear about this novel approach of fetal dissolution which greatly minimizes the maternal inconvenience of expulsion of the fetus.

In Table 4.14, the effect of 1 cc of double antigen containing dyptheria toxoid 0.5 cc and pertussis 0.5 cc intra amniotically in 14 cases of pregnancy with gestation varying from 10 to 16 weeks was observed. Here 42.85 % failure was noted on the seventh day and cumulative 28.51 % cases of failure on the 14th day.

The pathogenesis of diptheria is attributed to the release of a potent exdotoxin by the bacteria with a molecular weight of 55,350 dalton and with the help of trypsin it can be fragmented into two dissimilar fragments called A and B. Fragment A has a molecular weight of 21,150 daltons and if a single molecule gains entry into a cell it can catalyze ADP ribosylation of Elongation Factor 2 (EF2) using its nicotinamide adenine dinucleotide as a substrate. Ribosylation of EF2, which is essential for protein synthesis, leads to inhibition of proteins synthesis and death of cell which clinically manifests as necrotic lesion of dyptheria. When the dyptheria toxin is converted into toxoid, the immunogenicity is retained but the virulence is lost; however there may be rare reactions in the form of Type I (immediate type) hypersensitivity and delayed hypersensitivity [1].

The causative agents of pertussis are members of the genus Bordetella: they are small gram negative coccobacilli which are obligatory respiratory tract pathogens of warm blooded animals including birds. These are unable to survive outside their hosts. Four species are recognizable

Table 4.15 The effect of intra amniotic challenge of 2 cc 20 % bovine serum albumin on the growing human fetus up to 18 weeks

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen tetanus toxoid	Induction abortion interval range	Comments
28.2 ± 2.4 years	2.8 ± 6	14	11–18 weeks. Mean gestation with SD 14.6 ± 1.2	2 cc 20 % BSA antigen	77 h–12 days 4 h	8 patients did not abort on the 7th day. 4 patients did not abort on the 14th day

Table 4.16 The effect of maternal blood injection intra amniotically on the growing human fetus up to 14 weeks

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen schedule	Induction abortion interval range	Comments
24.4 ± 2.2 years	2.8 ± 6	10	8–14 weeks. Mean gestation with SD 13.4 ± 1.2	10 cc of maternal whole blood.	72 h–12 days 6 h	6 patients did not abort on the 7th day. 3 patients did not abort on the 14th day

and all of them excepting B. avium are pathogenic for human beings and can cause pertussis. B. pertussis is the most common agent for whooping cough. The pertussis toxin is synonymous with earlier known terms like histamine sensitizing factor, lymphositosis promoting factor, haemagglutinin, islet activating protein and pertussiger. It has a molecular weight of 117,000 daltons and consists of six polypeptides and two units, viz., a unit which is enzymatically active and the remaining five polypeptides cause the B unit which is needed to cross the membrane, eventually entering the cell and increasing the cAMP concentration and disturbing the normal cell function [1].

In the experiment relating to Table 4.15 a very simple antigen, namely 20 % BSA 2 cc, was injected intra amniotically in 14 cases with gestation varying from 11 to 18 weeks. Here 57.14 % of the cases did not abort on the seventh day and cumulatively 28.57 % cases did not abort on the 14th day.

We were extremely puzzled with the result. Initially we thought that the abortion is antigen specific, i.e., tetanus toxoid specific. Then in the course of our experimentations we concentrated on making the effect of induction to abortion predictable, i.e., abortion predictable within a fixed time. We did not achieve this goal, even after multiple injections or intrafetal injection of reducing the induction-abortion interval. Therefore we changed the antigen to BCG and saw a different effect altogether, i.e., fetal dissolution auto absorption. Due to ethical committee restrictions we used other antigens like double antigen (diptheria and pertussis) without any gross variation in the result. However, even with BSA, there was abortion, thus making the effect of tetanus toxoid non specific in nature.

Average fetal blood is 110 cc/kg which is approximately 50 % higher than the adult blood volume (75 cc/kg). Approximately 30 % of this circulating fetal blood resides in the umbilical cord and placenta. Thus, after excluding this blood the fetus has a net blood volume of approximately 80 cc/kg which is similar to the weight of normalized blood volume in lean adult humans.

If we look at Table 4.16, here too there is randomization of the weeks of gestation from the 8th to the 14th week. Here 10 cc maternal blood was injected into the amniotic fluid in ten cases. On the seventh day there was abortion in 40 % cases

Table 4.17 Intra amniotic leucocytes of 10 cc maternal whole blood

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen schedule	Induction abortion interval range	Comments cut off period 14th day
24.4 ± 2.2 years	3.1 ± 1. 6	16	9–15 weeks. Mean gestation with SD 12.6 ± 0.8 weeks	Buffy coat 10 cc of maternal blood	77 h–11 days 2 h	11 patients did not abort on the 7th day. 8 patients did not abort on the 14th day

Table 4.18 The effect of 5 cc freshly collected allogeneic amniotic fluid injection on the growing human fetus up to 16 weeks

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen schedule	Induction abortion interval range	Comments cut off period 14th day
26.4 ± 2.8 years	3.3 ± 1. 2	12	12–16 weeks. Mean gestation with SD 14.0 ± 1.8 weeks	5 CC of allogeneic amniotic fluid of the allogeneic mother	120 h–9 days 4 h	5 patients did not abort on the 7th day. 7 patients did abort on the 14th day

Table 4.19 Intra amniotic steroidin (Bacterial polysaccharide)

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen schedule	Induction abortion interval range	Comments cut off period 14th day
25.6 ± 1.4 years	3.4 ± 1. 2	10 cases	9–18 weeks. Mean gestation with SD 13.6 ± 2.4 weeks	Bacterial antigen (polysaccharide 2 cc)	56 h–11 days 8 h	6 patients did not abort on the 7th day. 4 patients did abort on the 14th day

The effect of 2 cc of bacterial polysaccharide injection on the growing human fetus up to 18 weeks

and on the 14th day 70 % cases aborted. Strangely, it appears that very little maternal blood (10 cc), can induce abortion in 70 % of the cases. This raises concern regarding certain serious questions of the critical amount of fetomaternal exchange in normal pregnancy and also regarding the problems of surgery on the unborn in the early weeks from the point of view of inadvertent fetomaternal exchange and the triggering off of abortion.

The effect of intra amniotic injection of buffy coated leucocyte collected from 10 cc maternal blood on the growing human fetus up to 15 weeks is shown in the Table 4.17.

If we analyze the above Table we see that with gestation varying from 9 to 15 weeks, injection of freshly prepared buffy coat of 10 cc of maternal blood, once, intra amniotically can trigger abortion (gestation randomized) in 31.25 % cases on the seventh day and cumulatively 50 % within 14 days.

In the experiment recorded in Table 4.18, we injected 5 cc of allogeneic amniotic fluid (single injection) to 12 cases carrying pregnancy from 12 to 16 weeks. Here too, seven cases (58.83 %) aborted within 14 days cumulatively and five cases (41.66 %) aborted within the seventh day.

In Table 4.19, we have used bacterial polysaccharide antigen 2 cc (steroidin) intra amniotically once in ten cases carrying gestations from 9 to 18 weeks; however, with stimulation of polysaccharide bacterial antigens, 60 % cases did

Table 4.20 To examine the effect of preservative and adsorbent in identical dosage in 2 cc blank (normal saline) on the growing human fetus

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen schedule	Induction abortion interval range	Comments cut off period 14th day
23.8 ± 4.4 years	2.8 ± 4	106	10–20 weeks. Mean gestation with SD 14.8 ± 1.8 weeks	2 cc of normal saline with thiomersol and aluminium phosphate	No abortion within 1 month in 102 patients. 4 patients which aborted on the 18th hour after injection and 42 h injection, 116 h and 22 h	All 4 patients had hype pyrexia and incomplete abortion. Amniotic fluid bacterial culture suggested haemolytic streptococcus and staph. Aureus in 3 cases, cervical culture positive for E.Coli in one case

Intra amniotic 2 cc of normal saline (with aluminum phosphate 0.01% and thiomersol 200 microgram) in the strength of tetanus toxoid

not abort on the seventh day and 40 % cumulative failure was noted on the 14th day.

As with other experiments discussed earlier, whenever there was slight/overt hemorrhagic tap during amniocentesis, the case was discarded from this study. In four cases there were incomplete abortions due to inadvertent infection and amniotic fluid culture was positive for haemolytic streptococcus, staph aureus etc. in three cases and cervical swab culture for E.Coli was positive in one case.

In Table 4.20, we have included 114 control cases where we brought specially prepared antigen blank ampoules from Chowgule e Hind, India Ltd, containing 2 cc of normal saline with thiomersol 0.01 % and aluminium phosphate 1 mg/ml. We waited after intra amniotic injection for 1 month to see if the injection prick itself or fetomaternal micro-exchange or maternal-fetus exchange or inadvertent trauma induced more exchange, i.e., fetomaternal transfusion or maternal fetal exchange, can cause abortion; and whether adjuvants like aluminium phosphate and preservatives like thiomersol can cause abortion. Eight cases were discarded from the study due to hemorrhagic tap and out of 106 cases there was abortion in 4 cases (3.77%). We are not precisely in a position to say whether fetomaternal exchange through the vent of placental or other fetal tissue at the site of amniocentesis or contamination of infection inadvertently or hematogenous infection from pre-existing maternal source or the adsorbent, aluminium phosphate itself, either singularly or in combination with preservative thiomersol, triggered the abortion process. The contributory role of materno-fetal transfusion is also not precisely known. And the last factor is that most of the control cases were injected in the year 1977–1984 when we did not have USG support to guide the amniocentesis.

In this connection, the reasons for *not using viral antigens*, may be mentioned. The institutional Ethical Committee suggested that the technical possibility/potentiality that attenuated virus may revert back to virulence in the hypoimmune or semi-immune or protected environment of the fetus. At least in case of rubella, the vaccine virus is capable of crossing the placenta and has been isolated from aborted fetal tissue from sites like the eye, kidney, bone marrow and the decidua. Whether the vaccine virus can damage the fetal tissue, as wild virus does in the case of adult tissue, is yet to be investigated.

In the experiment recorded in Table 4.21, 15 cases were included who opted for a second consecutive termination with tetanus toxoid. Here we find 86.66 % success rate on the 14th day. If we compare this with the first abortion scenario, the induction abortion interval does not show any gross variation.

Table 4.21 The impact of 2 cc tetanus toxoid on consecutive second pregnancy termination attempt through intra-amniotic route

Mean age in years with SD	Mean parity with SD	No. of cases	Weeks of gestation range	Antigen schedule	Induction abortion interval range	Comments cut off period 14th day
29.6 ± 4.2 years	3.1 ± 0.8	15	13–16 weeks. Mean 13.6 ± 1.4 weeks	2 cc of single fixed intra-amniotic injection	47.8 h–13 days 2 h (success rate is 86.66 %)	6 patients did not abort within 7 days and 2 patients did not abort within 14 days

Analysis of Table 4.22 shows that all the antigens used in the experiment in a fixed single injection schedule showed certain symptoms like aches and pain all over the body with or without mild pyrexia. This pyrexia mostly subsided after abortion, but very rarely it continued, viz., in tetanus toxoid single injection schedule, 112 cases (16.56 %) had aches and pain all over the body preceding abortion; and in 63 cases (9.15 %) the temperature was more than 99 °F, while in 1.01 % cases (7 cases) the temperature continued even after abortion. In control cases with normal saline, and adsorbant and preservative the incidence of pyrexia was 3.77 % (4 cases out of 106 cases). In the control cases where pyrexia continued even after abortion the amniotic fluid culture was found to be positive for pathogenic bacteria in three cases and cervical culture was positive in one case. In case of other antigens like sterodin, 20 % BSA, pertussis and dyptheria toxoid injection, the incidence of aches and pain all over the body varied like (a) 60 %, (b) 28.57 % and (c) 21.42 % respectively. With (d) whole maternal blood injections, (e) allogenic amniotic fluid injection, and (f) buffy coat leucocyte intra amniotic injection, the incidence of aches and pain varied, i.e., 40 %, 25 % and 31.25 % respectively. The overall incidence of aches and pain, if we randomize, was around 16.74 %. Now the question may be asked why there were aches and pains all over the body preceding abortion. In this connection certain trends may be noted.

From the onset of aches and pain all over the body the abortion takes place within 6–36 h. (mean 16.4 ± 4.6 h SD) and the abortion is inevitable. (As per WHO Prostaglandin Task Force Data Form, we gave a standard instruction to the on duty nursing staff not to give aspirin or other NSAIDs drug unless the pain was unbearable, for symptomatic relief, because of the theoretical possibility of delayed abortion and incomplete abortion). While the aetiology of aches and pains and temperature is not known to us in most of the cases as yet, the analogy lies in case of aseptic inflammation of myocardial infarction, where we find temperature due to release of cellular damaged products in the circulation like lysosomal enzymes etc.

A second question may be raised as to why aches and pains were present only in 16.74 % in all antigens. The answer is not straightforward. In the present series, most patients who volunteered for antigenic termination came from the lower socioeconomic strata, who were financially unable to afford prostaglandin or other methods. It has been found that such patients have a wide variation in the subjective tolerance of pain and ability to report its incidence to the attending nurse. We have included those cases where the pain was severe, i.e., unbearable, necessitating analgesic support like pethidine/morphine/pentazocin etc. In case of the BCG group this aches and pain syndrome necessitating request for analgesic support is noted in 79.92 %. Whether absorption of the fetal tissue, like the lysosomal content, is the cause of this generalized body ache is an important unresolved question for us. In this connection, during our limited unpublicized experience, two cases of selective fetal killing by (KCL) injection in the fetal heart (through USG) may be cited which also caused aches and pain to the mother within 48 h of fetal death.

Table 4.22 Showing the clinical events after intra amniotic antigenic challenge

Antigen used	No. of cases	Mean gestation	Aches and pain all over the body preceding abortion	Pyrexia	Pyrexia subsided after abortion	Pyrexia continued after abortion	Rupture of the membranes
2 cc 20 % BSA	14	14.6 ± 1.2	4 (28.57 %)	4	3	1	1
1 cc Dyptheria with pertussi	14	13.4 ± 1.4	3 (21.42 %)	3	2	Nil	1
10 cc of maternal blood	10	13.4 ± 1.2	4 (40 %)	2	2	Nil	Nil
Buffy coat of 10 cc maternal blood	16	14.4 ± 2.6	5 (31.25 %)	3	2	1	1
1 cc of Glutamate BCG single injection	26	13.2 ± 0.8	Instead of abortion there was fetal dissolution vis-avis auto absorption of the fetus (76.92) on the 14th day after 1 cc BCG injection (varying degree)				
Single injection 2 cc tetanus toxoid	688	13.6 ± 2.4	114 (16.56 %)	63	56	7	68* 70 fetuses were living at birth; all of them were 18 weeks or more. Not single fetuses aborted before 16 weeks and were living at birth
2 cc of normal saline with adjuvant and preservative	106	14.2 ± 1.2	4 cases had hyperpyrexia-amniotic fluid bacterial culture suggested staph aureas and haemolytic streptococci. It resulted in an incomplete abortion (3.77 %)				
2 cc of bacterial antigen Sterodin	10	13.6 ± 2.4	6 (60 %)	4	4	X	X** (One case had persistent bleeding after abortion necessitating 4 bottles of fresh whole blood transfusion and antibiotic and I.V. electrolyte solutions as a life saving gesture
5 CC amniotic fluid of the allogenic mother	12	14.2 ± 1.2	3 (25 %)	x	x	X	1
Total	896	14.6 ± 1.2	150 (16.74 %)	87	76	10	73

Post Abortion Maternal Effect

Table 4.23 suggests post-abortion mild hemorrhage, i.e., 1–5 days of bleeding (85.5 %): post-abortion moderate hemorrhage-6 to 10 days of bleeding (14.1 %); post-abortion severe hemorrhage -11 days or more of bleeding (0.4 %). Some blood loss during abortion is inevitable and this increases with the length of gestation. For the clinician there are two aspects of hemorrhage, i.e., an excess rate of blood loss or loss of an abnormally large volume of blood.

In all the cases the assessment is based on the subjective and objective coordination by the

Table 4.23 The distribution of cases by the amount of post-abortion bleeding after intra amniotic tetanus toxoid termination

No. of cases	Weeks of gestation	Mild hemorrhage	Moderate hemorrhage	Severe hemorrhage
32 Cases	8–11 weeks	21 cases	10 cases	1 case
336 cases	12–15 weeks	246 cases	88 cases	2 cases
186 cases	16–19 weeks	138 cases	48 8 cases	Nil
82 cases	20 weeks	78 cases	48 cases	Nil
82 cases	20 weeks and above	78 cases	3 cases	1 case

Table 4.24 The distribution of cases and histological correlationship of grade-II bleeding (5 days or more) after intra amniotic tetanus toxoid termination

Histopathology report after curettage	Retained product of conception	Fragments of deciduas only	Blood clots only	Others
48 cases	16	14	14	4

non-duty nursing staff and the resident doctor. In all cases, whenever the bleeding continued for more than 5 days, i.e., moderate bleeding, methergin (methyl ergometrin) support was started, and if inspite of methyl ergometrin support there was continuation of bleeding, evacuation of the uterus and a broad spectrum antibiotic (prophylactically) was advised (Ampicillin and Cloxacillin). In Table 4.23, out of 153 cases where the bleeding was more than mild, ergometrin support and clinical examination reduced the number of actual cases going for dilatation and evacuation (D & E) to 48 cases only. Clinical examination followed by ultrasound diagnostic support was utilized as and when necessary (Table 4.24).

The Table shows that out of 48 cases, 62.5 % of the cases showed some degree of conception material either in the form of retained product (16 cases, 33.3 %) or fragments of deciduas (29.16 %) at the time of histopathological examination.

Early resumption of normal menstruation vis-à-vis restoration of fertility, i.e., ovulation and reproductive function, is a serious concern for mothers who opted for abortion. The guilt and tension about abortion is largely abated with restoration of normal menstrual flow. For the clinician, the concern is the prevention of early complications, viz., hemorrhage, infection and uterine injury and not missing an ectopic pregnancy, leaving aside the problem of incomplete evacuation and infertility as late complications.

Table 4.25 Post-abortion maternal effect

Interval between abortion and the onset of first menstrual cycle	No. of cases	Gestation in weeks (randomized)
21–30 days	146	In 76.2 % of the cases menstruation returned within 40 days after termination with tetanus toxoid
31–40 days	136	
41–50 days	28	
51–60 days	46	
61-above		
	Total cases 356 cases followed up	

Distribution of cases on the basis of the interval between abortion and resumption of 1st menstrual cycle onset, after 2 cc fixed dosage intra amniotic tetanus toxoid termination protocol

The following few Tables show the follow-up study of abortion with (single fixed dose 2 cc) tetanus toxoid, with references to the menstruation of the patient. Here, too, all the cases which were terminated with tetanus toxoid did not turn up for follow-up study and advice inspite of our request and assurance of free treatment facility.

Therefore in the above Table 4.25, it appears that 76.2 % of the cases resumed their menstruation within 40 days.

Table 4.26 indicates that 58.1 % of the patients did not have any change in their period (when compared with the pre-abortion status) and

Table 4.26 Post-abortion maternal effect

Women followed up	196	(Cycles followed up: 784)
No change in menstruation	114 cases	=58.1 %
Regular to irregular cycles	16 cases	=8.8 %
Irregular to regular cycles	66 cases	=33.1 %

Behavior of menstrual cycle for the first 4 cycles after termination with intraamniotic tetanus toxoid single fixed dosage. (2 cc)

Table 4.27 Post-abortion maternal effect

Women followed up	176	(Cycles followed up: 528)
No change in cycle	146 cases	=82.9 %
Regular to irregular cycles	8 cases	=4.54 %
Irregular to regular cycles	22 cases	=12.56 %

Showing changes in their menstrual cycle, i.e., regularity for subsequent cycles, viz. 5–7th cycle after abortion with intra amniotic tetanus toxoid single fixed dose of 2 cc

33.1 % cases showed improvement, i.e., became regular from irregular bleeding (before abortion).

In Table 4.27, we have noted the fifth to seventh cycles after abortion and compared these with pre-abortional menstrual cycles. 82.9 % of our patients did not perceive any gross change in their cycle.

Table 4.28 shows the duration of menstrual flow for the first four cycles. Here we have noted on day 1, variation or no change in 49.4 % cases, and 2 days variations (decrease) were found in 33.77 % cases. However, 16.43 % of the cases suggested 2 days variation (increase) in their menstrual flow.

Table 4.29 depicts that when analyzing the subjective amount of menstrual flow, 59.1 % cases showed no change in the menstrual bleeding after abortion and 40.7 % cases showed marginal changes, i.e., slight increase or decrease of the menstrual amount.

Table 4.30 shows a history of dysmenorrhoea in 29 cases (before abortion). Out of 196 cases who reported for follow up clinic, in 11 cases, the dysmenorrhoea continued after abortion. Three cases who did not have a past history suggestive of dysmenorrhoea developed dysmenorrhoea after abortion with tetanus toxoid.

From Table 4.31, it appears that 32 cases, i.e. 16.32 % of the cases developed oligomenorrhoea to amenorrhoea after abortion with tetanus toxoid of which 2.04 % had amenorrhoea for 3 months follow up.

Table 4.28 Post abortion maternal effect: showing the changes in duration of menstrual flow for the first 4 cycles after intra-amniotic 2 cc tetanus toxoid termination

Women followed up	196	(Cycles followed up: 528)
No change in or 1 day variation (increase or decrease)	97 cases	=49.4 %
Duration of period increased by 2 days or more	33 cases	=16.43 %
Duration of period decreased by 2 days or more	66 cases	=33.77 %

Table 4.29 Post abortion maternal effect

Women followed up	196	(Cycles followed up: 784)
No change in amount of periodic bleeding (increase or decrease)	116 cases	59.1 %
Increase in the amount of periodic bleeding	42 cases	21.4 %
Decrease in the amount of periodic bleeding	38 cases	19.3 %

Single fixed dose (2 cc) abortion with intra amniotic tetanus toxoid showing changes in amount of menstrual flow in the first 4 cycles

Table 4.30 Post abortion maternal effect

No. of cases followed up = 196 (No. of cycles followed up = 784	
History of dysmenorrhoea before abortion	29 cases
Continuation dysmenorrhoea after abortion	11 cases
Development of dysmenorrhoea after abortion (no previous history)	3 cases

Showing incidence of post-abortional dysmenorrhoea in cases of intra amniotic tetanus toxoid termination with fixed single dose schedule. (2 cc)

Table 4.31 Post abortion maternal effect

No. of cases followed up:	'96	
No of cycles followed up	588	
Incidence of oligomenorrhoea	28	14.28 %
Incidence of amenorrhoea	4	2.04 %

Showing the incidence of amenorrhoea to oligomenorrhoea (subjective assessment in comparison with the pre-pregnancy bleeding) after termination with single fixed intra amniotic dosage (2 cc) injection protocol with tetanus toxoid, follow up for 3 months

Table 4.32 Post-abortion maternal effects

No. of patients studies = 88, (Sequential-12th, 14th, 16th, 18th day folliculometry)		
Ovulation present	51	57.95 %
Ovulation absent	15	17.04 %
Ovulation doubtful	22	25 %

Single fixed (tetanus toxoid) intra amniotic injection schedule and post-abortion follow-up showing detection of ovulation by sequential folliculometry on the first menstrual cycle

Table 4.33 Post-abortion maternal effects

No. of patients	36
No. of cycles	144
Ovulation present	32 (88.88 %)
Ovulation absent	2 (5.55 %)
Ovulation doubtful	2 (5.55 %)

Single fixed (tetanus toxoid) intra amniotic injection schedule and post-abortion follow-up showing folliculometry on the first 4 menstrual cycles (up to 7 months)

The above two Tables (4.32 and 4.33) show resumption of ovulation with tetanus toxoid termination (fixed dose schedule) as per the report of sequential USG examination from the 12th to the 18th day after period. Ovulation is noted in 57.95 % cases out of 88 cases who reported for this study in the first month of termination and 88.8 % cases had folliculometry suggesting ovulation within the first four cycles (up to 7 months).

Future Pregnancy Potential

The vast majorities who have a safe induced abortion experience suffer no long term effects on their general or reproductive health. The exceptions are a small number of women who have severe, immediate or delayed complications like secondary infertility, ectopic pregnancy, miscarriage and a fear of adverse effects on the subsequent pregnancies like low birth weight, pre-term labour, fetal abnormalities, placenta previa, psychological effects and fear of cancer.

Table 4.34 Post-abortion maternal effects. Future pregnancies follow up termination of a fetus with intra amniotic tetanus toxoid

No. of cases studies = 146 (post tetanus toxoid termination of pregnancy)	
a. IUGR	6 (4.10 %)
b. Gestosis (PET only)	14 (9.58 %)
c. Gestosis+IUGR[a]	4 (2.73 %)
d. Ectopic pregnancy	Nil
e. Placenta previa	Nil
f. Miscarriage	1
g. Pre-term labour	1

IUGR Intrauterine growth retardation, *PET* Pre-eclampsia toxemia

[a]Very poor nutritional background and poor compliance with antenatal advice

Table 4.34 shows a follow up of 146 cases with the age of the mother randomized. The incidence of idiopathic IUGR without any obvious reason is 4.10%, i.e., six cases. The incidence of specific conditions like edema (E) proteinuria (P), hypertension (H) syndrome specific to pregnancy (EPH Gestosis or toxemia of pregnancy) is 9.58 % of the total cases. Again another 2.73 % cases had IUGR with pre-eclamptic toxemia. Here too, any specific conclusion would be too simplistic because of age, parity, nutritional status randomization.

If we analyze Table 4.35, 26 cases were randomized who were enrolled for auto immune study. We have noted that 11.53 % cases had marginally positive anti cardiolipin antibody titre both IgG and IgM (positive) and 7.69 % cases had a positive anti nuclear antibody. Another 7.69 % cases had a positive report for lupus anti coagulant and 3.84 % cases had a positive anti double strand DNA antibody. However, except in one case where the complement reactive protein continued to be positive even after 3 months, the other auto immune parameters became negative. On closer scrutiny of past history and through

Table 4.35 Immuno-endocrino-hematological impact of intra amniotic antigenic challenge to the mother with tetanus toxoid

Auto immune study	No. of cases	Normal range	Positive result	Percentage
Complement reactive protein (CRP)	26	Less than 6 mg/ml	1	3.84 %
Anti nuclear antibody	26	1:40-1:80 above significant	2	7.69 %
Anticardiolipin antibody (IgG and IgM)	26	Anticardiolipin antibody. IgG up to 15 GPL units/ml		
IgM up to 12:5 MPL units/ml	2	11.53 %		
Lupus anti coagulant	26	Negative	2	7.69 %
Anti double strand DNA antibody	26	1:10 and above is significant	1	3.84 %

Post-abortion maternal effects: Follow-up immunological study after 1st pregnancy termination with tetanus toxoid showing residual reaction of the mother to this new method of abortion

Table 4.36 Auto immune profile of mother during her consecutive second pregnancy termination with intra amniotic tetanus toxoid showing long term reactions of the mother in case of 2nd pregnancy termination (consecutive with intra amniotic antigenic challenge tetanus toxoid)

Auto-immune study	No. of cases	Range (normal)	Result (positive)
Complement reactive protein (CRP)	12	Less than 6 mg/ml	2 (16.66 %)
Anti nuclear antibody	12	1:40–1:80 above significant	1* (8.33 %). Report became negative on 3 months follow up
Anticardiolipin antibody (IgG and IgM)	12	IgG up to 15 GPL unit/ml. IgM up to 12.5 MPL units/ml	1* (8.33 %). Report became negative on 3 months follow up
Lupus anti coagulant	12	Negative	1* (8.33 %). Report became negative on 3 months follow up
Anti DNA antibody (double strand)	12	1:10 and above is significant'	1* (8.33 %). Report became negative on 3 months follow up

clinical examination of the same patient, she was found to be suffering from rheumatic arthritis and her ASO titre was found to be high.

From the above experiment pertaining to Table 4.36, it appears after a second consecutive termination with tetanus toxoid, there was no residual unusual autoimmune activity in the mother; 8.33–16.66 % of the cases which showed a positive titre became negative at 3 months follow up.

If Table 4.36 is analyzed, 8.33 % had a positive anti nuclear antibody, anti phospholipid antibody (anti cardiolipin antibody), lupus anti coagulant, anti- DNA antibody (double strand) which became negative at the follow-up study in the third month. However, 16.66 % of the cases showed a positive complement reactive protein which also became negative in the 3 months follow up study.

In the experiment relating to Table 4.37, we wished to examine whether sharing of DR/DQ triggers early abortion, i.e., within 72 h of first trimester cases only. Though the series was small (N = 10), we did not find any correlation between the parents' HLA DR/DQ studies and early abortion. Hence, accidental or chance sharing of HLA DR/DQ is possibly not a cause of early abortion. Rather, the intra amniotic tetanus challenge may be the possible trigger for the abortion process.

In Table 4.38 we find maceration of the fetus more in the below 17 weeks gestation group, viz., 48 cases showed maceration (58.53 %) and 9 cases did not show any clinical maceration (10.97 %); here again the male fetuses were 66 % in the macerated group and 22.22 % in the non-macerated group. In cases of fetuses above 17 weeks, 8 % cases were found to have maceration with 4 % each from each sex.

Table 4.37 Paternal and maternal HLA DR/DQ study of the 1st trimester early abortions (within 72 hours after intra amniotic single dosage, fixed tetanus toxoid termination)

		HLA type class II				
		DR	Locus	DQ	Locus	Others
Case I	Father	15 (27)	–	1	–	–
	Mother			2	–	DR 53
Case II	Father	3	–	1	–	DR 52
	Mother	7	17 (3)	–	–	DR 52
Case III	Father	15 (2)	17 (3)	1	2	DR 53
	Mother	7	–	3	–	DR 52
Case IV	Father	15 (2)	17 (3)	1	–	DR 53
	Mother	7	–	2	–	DR 52
Case V	Father	15 (2)	–	2	–	DR 53
	Mother	7	–	1	–	–
Case VI	Father	3	–	1	–	DR 52
	Mother	7	17 (3)	–	–	DR 53
Case VII	Father	1 (03)	–	1	–	DR 52
	Mother	7	–	4	2	DR 52/53
Case VIII	Father	11 (5)	–	2	–	DR 52
	Mother	7	17 (3)	3	–	–
Case IX	Father	1	15 (2)	3	–	–
	Mother	7	15 (2)	1	–	DR 53
Case X	Father	14 (6)				
	Mother					

However in the non-macerated group above 17 weeks, 56 % cases were found to be female fetuses and 36 % were male fetuses.

In Table 4.39, from the macroscopical study of the abortus, it appears that the fetus, if it is above 17 weeks or more, the degree of maceration abruptly decreases. Whether this is the result of the appearance of the HLA system in the growing fetus or due to some other factor like maturation and growth of the organs, is a problem to be solved in the future. The other theoretical possibility is the delay in expulsion of the fetus after its death. Is this the result of the immaturity of the fetal system or the inability of the uterus to expel the dead fetus? This is also a problem to be solved in the future. There is also the third possibility of non-fetal participation, due to death, in the labour initiation process; however, the most important clue may be that fetal death is not immediately followed by placental non-function, delayed expulsion and the reverse, i.e., placental dysfunction followed by expulsion or death of the fetus, in case of early expulsion of the fetus. In case of pre- HLA (before 17 weeks), the fetal death may precede in most cases of abortion and with progressive maturity of the fetus, placental dysfunction may precede the expulsion/death of the fetus. If the expulsion is earlier, the fetus may be living, depending on the state of gestational maturity of the fetal organs.

What Is Our Ultimate Learning from This Search for a Safe, Effective, Cheap Midtrimester Abortifaecient

Combating the menace of unsafe or criminal abortion in the developing world in particular, by the intra amniotic injection of 2 cc tetanus toxoid to mothers who want an abortion could be a good and rational suggestion for our unfortunately poor patients who want a safe abortion.

Adequate popularization of this simple, safe, cheap and easily procurable, effective method of abortion [1–24] can combat the criminal abortion menace. In the developing countries in particular,

Table 4.38 Single fixed dosage schedule (tetanus toxoid) and the state of expelled fetuses in terms of gestation

No. of cases	Gestation up to 16 weeks (10.99 %)				17 weeks and above macerated 8 %				Comments
	Macerated (58.53 %)		Non macerated		Macerated		Non-macerated		
	Male	Female	Male	Female	Male	Female	Male	Female	
82	32 (58.53 %)	16 (58.53 %)	2 (10.97 %)	7 (10.97 %)	1 (2.43 %)	1 (2.43 %)	9 (28.04 %)	14 (28.04 %)	Living fetus at birth 17 weeks and above (non macerated) 23 cases; before 17 weeks group: 6 cases were living at the time of abortion

Table 4.39 Single fixed dosage schedule (tetanus toxoid) induced maceration and the induction – abortion interval co-relationship

No. of cases	Weeks of gestation	Mean induction abortion interval with SD	No. of macerations	Maceration of the fetus	Living fetus or dead fetus at abortion
9	8–11	96 ± 24.6 h SD	X	9	29 living fetuses were seen at birth. 53 dead fetuses were seen at birth
42	12–15	118 ± 32.4 h SD	18	39	
20	16–19	144 ± 48.6 h SD	11	2	
11	20 above	186 ± 36.4 h SD			

local health departments can take the initiative by organizing camps and workshops and encouraging the participation of social workers, nurses, midwives, village level quack doctors or barefoot doctors, pharmacists or even criminal abortionists agreeable to accept change, from any sphere of life in urban, semi-urban and rural areas. Inadvertent intramuscular (intra-uterine musculature) injection of the mother will sensitize and boost the maternal existing antibody against tetanus. If the mother in such a case decides to continue the pregnancy after 18 weeks the mother would be protected against neonatal tetanus with a subsequent injection after the first month. Global mortality from tetanus is 1 million of which 880,000 deaths are recorded for neonatal tetanus [1–25].

Reaction of the Pre-immune or Hypo-immune Fetus (up to 20 weeks)

Earlier investigations reported the implications of congenital infections in case of maternal syphilis [26] and rubella in the human system [27–32], rubella infection and blue tongue virus infections in lambs and lymphocytic choriomeningites virus in mice [33]. They suggested that the developing fetus of many mammalian species is able to mount a highly efficient immunological response to the agents responsible for congenital infections.

The present research is the first documented work (Medline search verification, 24 October 2014) on intra-amniotic direct antigenic challenge to the human fetus showing the reactions of the developing pre-immune and hypo-immune fetus; however, there are certain obvious differences with the congenital infection scenario.

In case of congenital infections, the placental barrier is formidable and unless this is broken, access to the fetal compartment or fetal organs is not possible. The sequence of events are maternal severe infection, involvement of placenta and loss of placental function – in all these, the fetus is involved. The damage to the fetal system is due to the resultant action of toxins/chemicals liberated by the living or dead/offending organisms and their interaction with the growing fetal system as well as the primitive non-specific to specific developing immunological defense against the offending organisms.

In case of our experimentation with different antigenic challenges, the placenta is never damaged primarily. It is only damaged secondarily to fetal damage or death. Hence the changes we find are primarily the result of fetal changes due to the interaction of the fetal system with the antigenic stress. On the basis of macroscopic and microscopic studies, we found similar hitherto unknown fetal reactions to an antigenic invasion of the fetal system: to 20 % bovine serum albumin 2 cc, 10 cc maternal whole blood, collected from leucocytes or buffy coated layer collected from 10 cc maternal blood, allogeneic amniotic fluid 5 cc of other mothers, bacterial antigen like sterodin, double antigen, tetanus toxoid, etc.

However, the reactions are much more with glutamate BCG (up to 15 weeks). Here fetal death and fetal dissolution was observed of varying degrees without abortion, a hitherto unknown

phenomenon (vide Medline search covering the last 40 years).

If we analyze the sensitivity of the growing human embryo to fetus in the light of our experiments with intraamniotic challenge, we can observe the following:

(a) Intra amniotic tetanus toxoid antigen challenge in the teratogenic phase (up to 9 weeks) to the pre-immune phase of human fetal growth (10–16 weeks) leads to massive hemorrhage; also congestion of all the viscera and changes in the growing architecture of the fetal organs, eventually leading to death and abortion of the fetus.
(b) Tetanus toxoid challenge at the hypo-immune phase (17 weeks onwards) of human fetal growth leads to mono cellular cell invasion, hemorrhage, thrombosis in all the organs including the placenta where there are additional features of villites and deep subchorionic fibrosis, eventually leading to abortion in a less dramatic way with mostly living fetuses.

Hence, we can say that even in the sterile environment of the amniotic fluid, the developing fetal system can also react to any antigenic challenge; however, the reactions are dependent on type/route/virulence/dosage of the antigenic assault. It is believed that this at least will partially explain the apparent benign presence of treponema palladium over the very early fetus [26]. On the basis of research conducted over the last three to four decades it is felt that a little more presence of treponema antigenic load in a very young fetus will abruptly change the entire scenario from benign presence to a cruel presence triggering death cum deformity and abortion of the fetus depending upon the stage of fetal growth from the teratogen to the pre-immune phase or hypoimmune phase of the fetus due to the crossing of the critical mark of fetal susceptibility cum tolerance of the antigen load. This may result in an exaggerated and hypersensitive type of acute response in the early weeks (up to 16 weeks) and a chronic response in the later weeks (above 17 weeks) as seen with different antigenic challenges which is evident clinically in the case of the aborted fetus, depending on the gestational age of the fetus.

The above were the research findings regarding the development of different parameters for growth and differentiation of the human fetus from 8 to 20 weeks time.

It is well known that the function of the immune system is to protect the individual from invasion of foreign antigens by distinguishing the self from the non self. A normal immune response relies on the careful coordination of a complex network of specialized cells, organs and biological factors necessary for the recognition and subsequent elimination of foreign antigens. An exaggerated immune response can result in the hypersensitivity to foreign antigens with resultant tissue injury and the expression of a variety of clinical syndromes like Type 1 (IgE mediated) immediate hypersensitivity reaction, Type II (IgG, IgM, complement activation) antibody mediated cytotoxicity reaction, Type III (antigen antibody complex and complement activation) immune complex reaction and Type IV (lymphocyte) delayed hypersensitivity reaction. Two other mechanism are also proposed: Type V and Type VI, of which, Types I–III are humoral immune responses and Type IV response results from initial sensitization and subsequent antigenic challenge. This kind of antigen elimination through the cellular or humoral process is integrally linked to the inflammatory response in which the cellular messengers (cytokines) and antibodies trigger vaso active inflammatory mediators. This inflammation has got a positive role, i.e., to promote efficient elimination of foreign antigens and to prevent uncontrolled lymphocytic activation and antibody production and also negative and deleterious effects. In the case of the growing innocent embryo or the hypo immune human fetus, all the factors responsible for inflammatory response and its coordination are also developing at that stage of gestational maturity. Hence, poor coordination may lead to the negative effect of inflammation due to inappropriate activation in a growing fetal system thus causing dysregulation and perpetuation of the inflammatory process which leads to tissue damage and organ dysfunction and the death of the fetus with massive hemorrhage and congestion of all the viscera (up to 16 weeks).

From 17 weeks onwards, due to the progression of the ontogeny of the human immune system, the reactions are less dramatic, typified by the characteristic changes of mononuclear invasion, less hemorrhage and congestion and damage to the architecture of the growing human fetus. Researchers from immunology to endocrinology and from gynaecology to biochemistry should unite together and sit together to standardize the reactions because these have immense implications for fetal organ transplants and surgery on the unborn, apart from the problem of fetal tissue transplant in the adult system. In this connection, umbilical cord blood stem cell transplants are extremely encouraging because of its hypo-immunogenicity and no graft versus host reaction when the umbilical cord blood stem cells are transplanted into the adult system.

Implications of This Type of Research in the Field of Fetal Surgery and in Understanding the Cause behind Premature Labour in Cases of Surgery of the Unborn

Powerful imaging and sampling techniques have stripped the veil of mystery from the once secretive fetus. Although most fetal defects are best managed after birth, a few predictable life threatening developmental consequences have been successfully corrected in utero; however pre-term labour remains a significant risk to both the mother and the fetus [34].

Our experience with maternal 10 cc blood injection or leucocytes of 10 cc maternal blood injection through the intra-amniotic route and even a non-specific antigenic challenge like BSA 20 % 2 cc shows that these can trigger abortion in 71.4 % of cases within 14 days. Hence, we believe that the most important problems in surgery of the unborn is feto -maternal exchange vis a vis sensitization which can initiate premature labour, whereas materno-fetal exchange can cause death, deformity, abortion as well as premature labour depending on the antigenic load, period of exposure and gestational maturity of the fetus at that point of time.

We also wanted to remind all investigators in the field of the various theories in the pathogenesis of vascular disrupting syndromes following the commonly practiced chorionic villous sampling (CVS) for detection of congenital abnormality with the initiation of a vascular incident after CVS with thrombosis at the sampling site, may result in (a) atrophoblast embolism and embolisation of fetal vessels, resulting in hypoperfusion of fetal circulation followed by voluminous fetal maternal transfusion or release of vaso active substances [35–38], (b) damage of the extra amniotic tissue, especially the fetal membranes with subsequent limb malformation due to amniotic band of oligohydramnios [39–41] or (c) entrapment of limbs to the extra coelomic space [42, 43]. However, the studies referred to above emphasized on the non-immunological link of a vascular disrupting syndrome. Maternal sensitization due to inadvertent fetomaternal hemorrhage which can be followed by materno-fetal hemorrhage as a compensatory exchange transfusion has been noted by AH Lipson and WS Webster [44]. On the basis of our experience the two hit phenomenon of an immunological model as suggested by AH Lipson and WS Webster [44] in case of fetomaternal surgical operations on the unborn as a triggering factor for premature labour. Hence, it is suggested to practice extreme caution and incorporation of micro surgical procedures to minimize the risk of fetomaternal vascular disruption for mothers undergoing surgery on the unborn fetus, resulting in sensitization of the fetus vis-à-vis the mother or vice versa and its implications on the mature immunological system (the mother's) and the immature immunological system (the fetal) and its overall impact on the initiation of abortion or premature labour, depending on gestational maturity.

Future of Hematopoietic Stem Cell Transplant in Utero

A variety of congenital defects, potentially curable by hematopoietic stem cell transplantation are now feasible in early gestational anomalies like immunodeficiencies, haemoglobinopathies

and storage diseases. However, there are problems related to donor availability, graft vs host disease, graft rejection in case of adult bone marrow.

The alternate sources of hematopoietic stem cells are either the liver from an aborted fetus or the neonatal umbilical cord blood. In the case of stem cells collected from the liver of a pre-immune less than 15–16 weeks fetus, there is no problem of graft versus host disease; hence T cell depletion is not necessary [45] there is the added advantage of homing as they are primed to travel to the waiting fetal marrow and the fetal cells then can be reprogrammed to a lifetime of self-renewal. Investigators already transplanted hematopoietic stem cells in a fetal sheep and monkeys [46, 47] and demonstrated long lasting hematopoietic chimerism of all cell lines without rejection, host versus graft disease or need for marrow ablation or immunosuppression. Umbilical cord blood stem cells may also be an attractive alternative source of hematopoietic stem cells for adult patients. The observation that is most intriguing is the low incidence of acute or chronic graft versus sot disease [48], regardless of the HLA background or the pregnancy induced maternal tolerance or the blunted proliferation response to neo antigens and T cell cytotoxicity of the umbilical cord blood stem cells [49] is responsible for less graft versus host response in case of pediatric or adult transplantation is a matter under intense scrutiny by the scientific community.

On the basis of human ethically permitted volunteer research on the disruption efficacy of intra amniotic antigenic challenge on the pre-immune fetus up to 15–16 weeks with 2 cc bovine serum albumin 20 %, WBC of 10 cc maternal whole blood, maternal 10 cc whole blood, 5 cc allogeneic amniotic fluid of other allogeneic mothers, it is noted by us that there is abortion of the fetus in varying percentages within 14 days. We also confirmed to the entire scientific community working on the problem of surgery on the unborn that caution should be exercised and that antigenic challenge even to the pre-immune fetus can cause hitherto unnamed fetal reactions, leading to death and abortion of the conceptus. Extreme caution should be exercised at least in the case of hematopoietic stem cell transplantation in utero to combat and cure genetic diseases because of the negative impact of inflammation type responses in the growing phase of the embryo or the fetus [50–55] (Figs. 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, and 4.10).

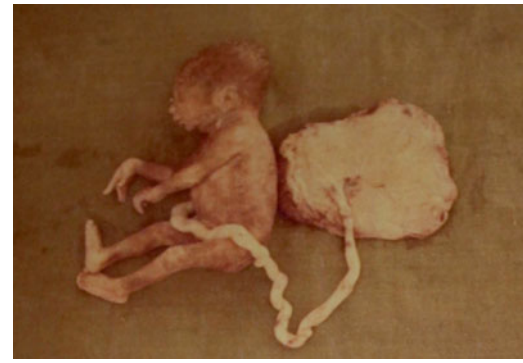

Fig. 4.1 Shows a non macerated fetus of 18 weeks gestation. Softening of the viscera is noted. There is deep subchorionic fibrosis or whitish appearance of the placenta

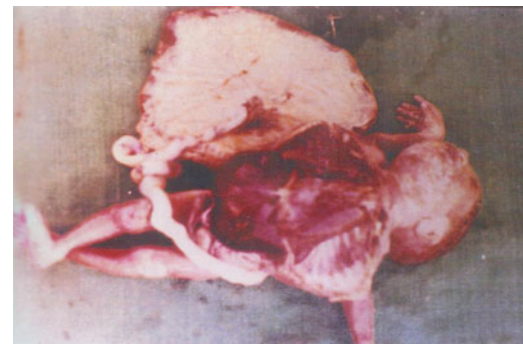

Fig. 4.2 Shows a fetus and placenta at 19 weeks. Here the placenta showed whitish, deep sub-chronic fibrosis. Here, the abortion took place on the 11th day after intra amniotic instillation of tetanus toxoid

Conclusion

What has not been done yet but essentially needs to be done in the future to find a solution to the many questions raised regarding the issue of antigenic challenge to the growing human fetus in utero is listed below:

(a) If we consider pregnancy as a balance of Th1/Th2 response [55], sequential study

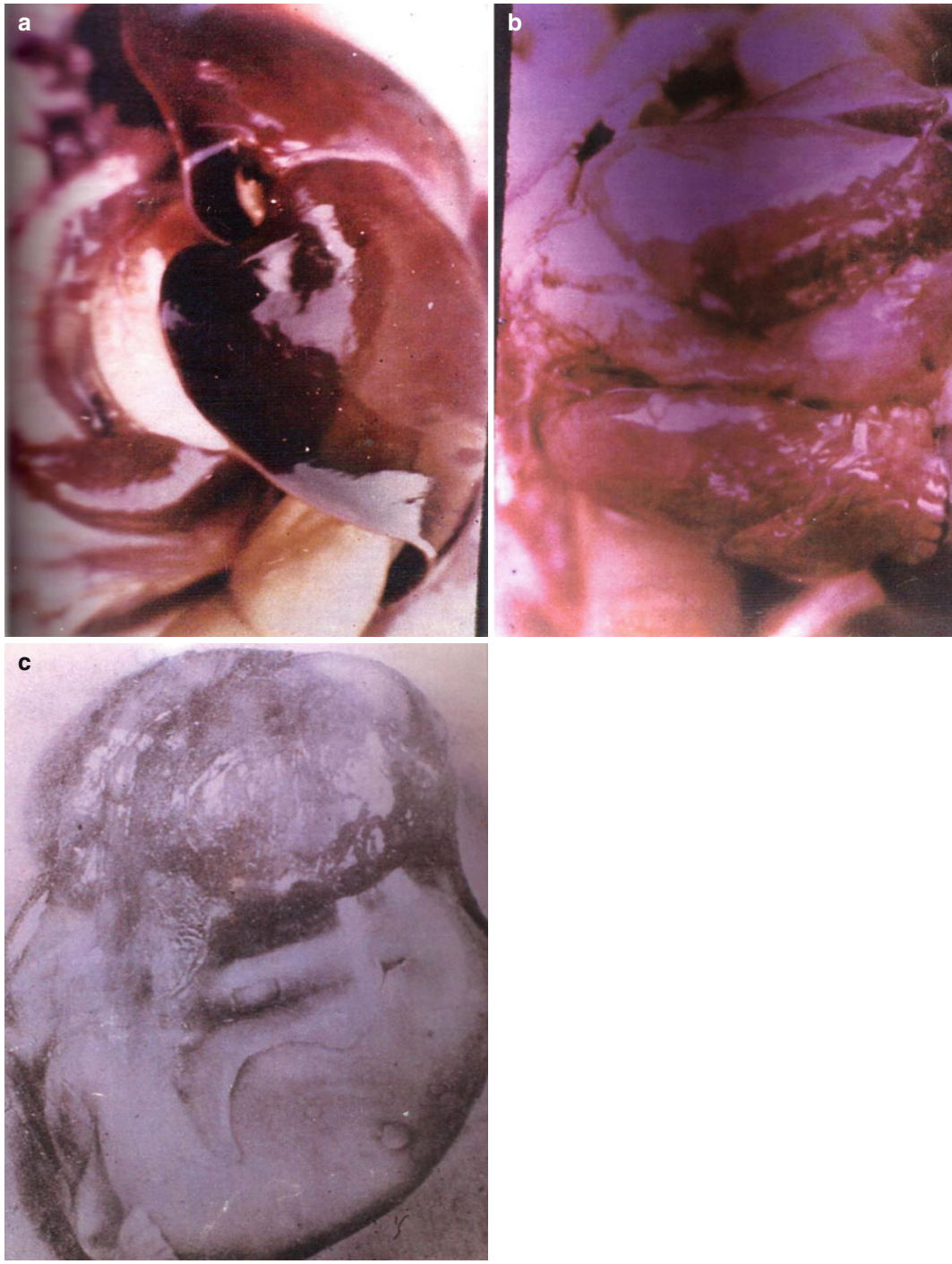

Fig. 4.3 (**a**–**c**) This is a unique representative photograph showing a fetus (18 weeks) expelled after intra amniotic instillation of (2 cc) tetanus toxoid on the seventh day. The important aspect of the photograph is the fact that the entire fetus, placental membrane and the amniotic fluid inside the amniotic sac came out simultaneously, justifying the abortion as complete. Our coagulation studies (vide text) had proved that there is no fall in platelet, fibrinogen and a change of fibrinogen degradation products with this method of abortion. In this connection it is worth mentioning that the price for 2 cc tetanus toxoid is Rs. 4 (Four Indian Rupees only which is equivalent to Five American cents)

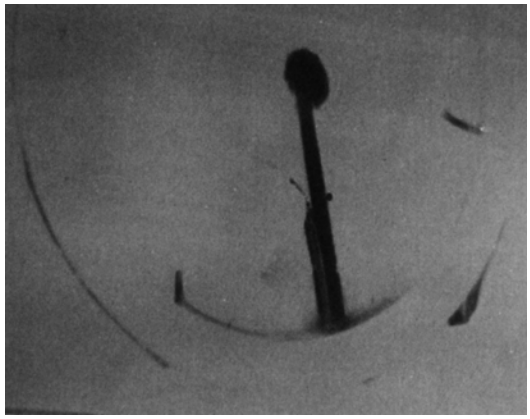

Fig. 4.4 The migration inhibition of fetal thymocytes noted in the presence of tetanus toxoid antigen, when tetanus toxoid injection (three injections at 1 month interval) was given intra muscularly to the mother (18 weeks pregnancy)

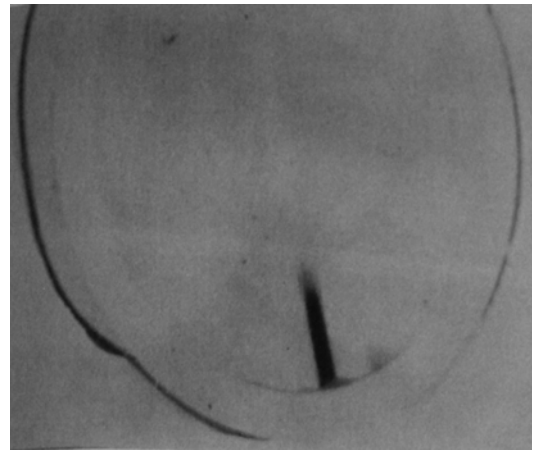

Fig. 4.6 Depicts the effect of intra amniotic antigen (tetanus toxoid) on migration of fetal thymocytes (17 weeks) on the 14th day

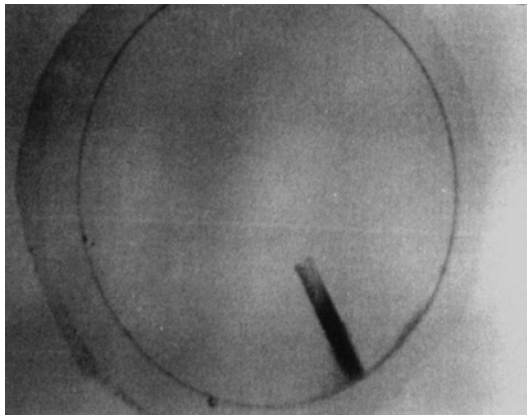

Fig. 4.5 Migration inhibition of fetal thymocytes noted in the presence of tetanus toxoid antigen, after 14 days of intra amniotic tetanus toxoid injection (18 weeks)

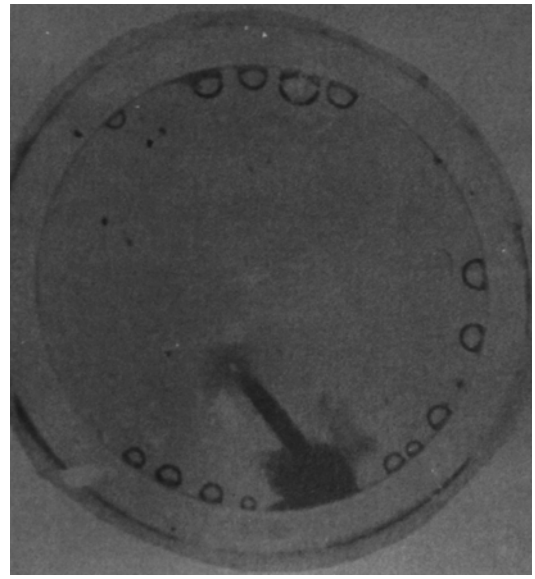

Fig. 4.7 Depicts the effect of intra amniotic antigen (tetanus toxoid) on migration of fetal thymocytes (17 weeks) on the seventh day

of the Th1 mediator (tumor necrotic factor, gamma interferon etc.) and Th2 mediators after intra amniotic challenge, would provide an exact understanding of the cellular action involved in abortion.

(b) A study of TJ6 protein of the fetal thymus after antigenic challenge as well as maternal blood lymphocytes sequential TJ protein binding [56–58] estimation will help us to understand the fetomaternal impact of intra amniotic antigen challenge.

(c) Sequential fetal immunophenotypes after the antigenic challenge to the growing fetus help to answer many unanswered questions related to this research work, particularly in the area related to fetal growth and maturation and other specific responses of the fetus with antigenic challenge.

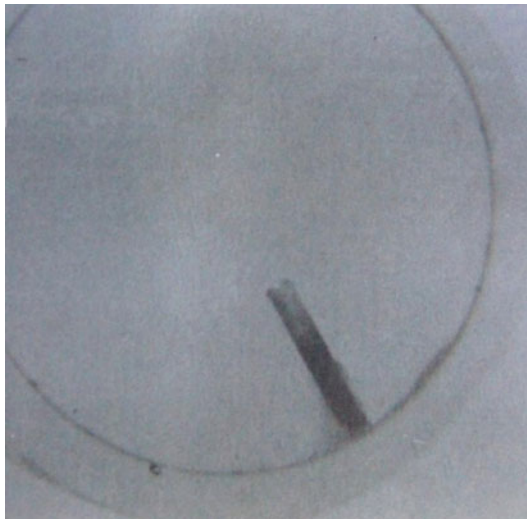

Fig. 4.8 Depicts the effect of intra amniotic antigen (tetanus toxoid) on migration of fetal thymocytes (17 weeks) of fetal thymocytes (17 weeks) on the 21st day

(d) Sequential specific immunoperoxidase staining of the fetal and maternal leucocytes after intra amniotic antigenic challenge and its interactions will boost the existing field of knowledge in the field of fetomaternal interactions after intra amniotic antigenic challenge in pregnancy.

Acknowledgement I gratefully acknowledge the guidance of the Ethical Committee headed by late Prof KP Sengupta, PhD, Director, IPGMER, SSKM, Calcutta, India. I am also grateful to Dr. MK Chhetri, Director of Health Services (retired) for his guidance. I sincerely acknowledge and thank all my patients for their wholehearted objective support and trust in our research which entails the search for a safe, cheap and effective abortifaecient.

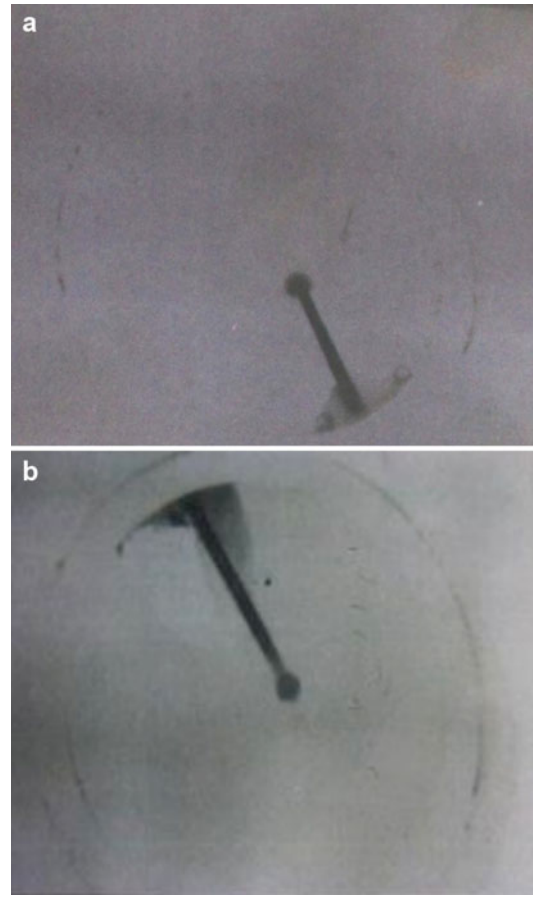

Fig. 4.9 (**a**, **b**) Tetanus toxoid injected I.M in the above two pictures to the mother does not show migration inhibition of fetal thymocytes. We have seen in the preview of the present study that antigen (tetanus toxoid) stimulation of the mother causes liberation of MIF factors, but maternal sensitivity with antigen has got no effect on the fetal system and vice versa, i.e., fetal antigen sensitivity has got no impact on maternal system up to 20 weeks of human fetal growth. We can suggest that the impact of antigen possibly depends on gestation, maturity, exposure and amount of antigen in a developing immune system

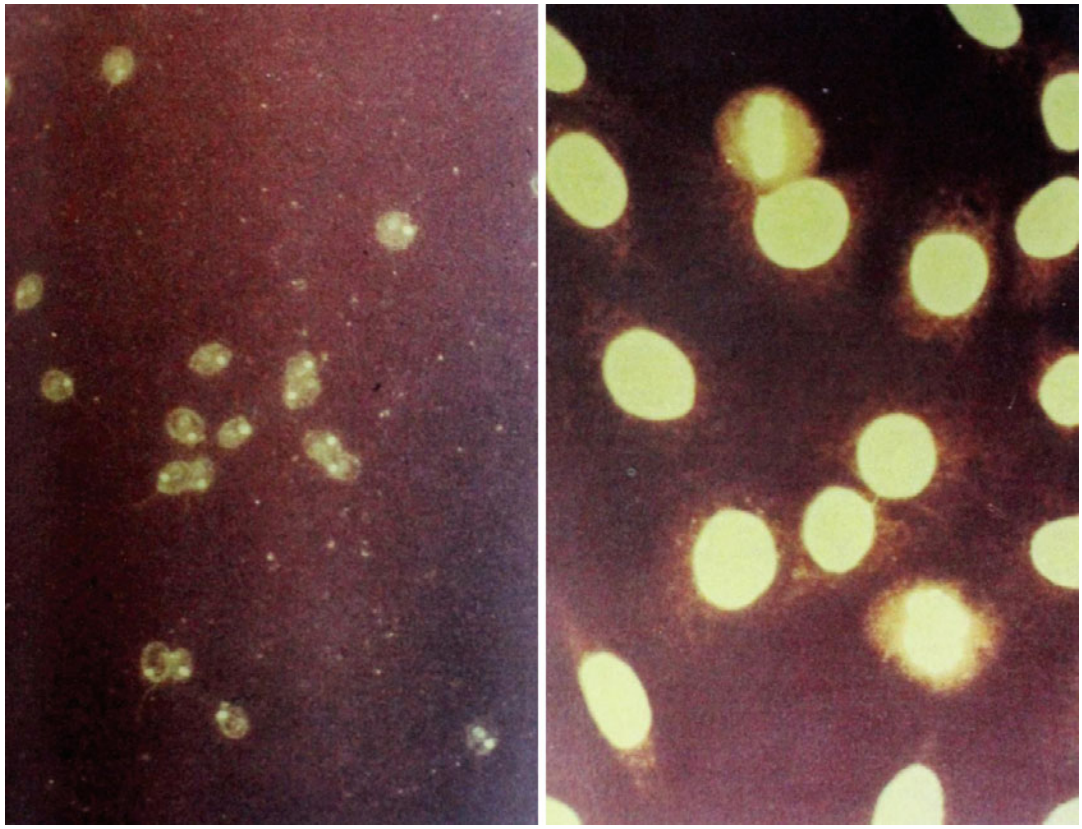

Fig. 4.10 Immunoflourescence study was done to reconfirm positive cases only and document autoimmune reactions, i.e., DS (DNA), and of ANA, of a mother undergoing abortion with intra amniotic antigen, though routinely all cases were done by ELISA (Enzyme Linked Immunosorbent assay) for semi-quantitative detection of auto antibodies

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Fetal Growth and Development in the First Two Trimesters

5

Aditi Aikat, Tarun Kumar Roy, and Niranjan Bhattacharya

The story of human life begins much beyond birth…

As soon as an oocyte is fertilized by a sperm, a journey ensues from the totipotent zygote undergoing cell division, cellular migration, differentiation, apoptosis (programmed cell death), growth and cell re-arrangement that involves temporal and spatial control of gene expression guided by epigenetic parameters, to evolve into an organised multi-cellular human being.

The word fetus (plural fetuses) is from the Latin fetus, "the bearing or hatching of young, a bringing forth," from Latin base *fe- "to generate, bear," also "to suck, suckle" [1]. The fetal period extends from ninth week of gestation, the first 8 weeks being the embryonic period, during which the embryo gradually transforms into a fetus with the primordial characteristics of all major systems having formed and bearing a recognizable human appearance. The development in the fetal period is primarily concerned with rapid body growth and differentiation of tissues, organs and systems, while the rest of the body takes over growth rate of the fetal head deviating from the embryonic period where there is a head start regarding growth rate [2].

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First Two Trimesters

The first two trimesters, which are each a period of 3 months are again unique with all the major systems getting developed in the first trimester itself and the second one seeing the fetus gain sufficient growth size-wise with detailed visualization possible through USG [3]. The fetus during this period exhibits the unique characteristic of healing without scar, having a critical balance between Matrix metallo proteinases (MMPs) and Tissue Inhibitors of MetalloProteinases (TIMPs) [4], demonstrating lack of fibroplasia, increased Hyaluronidases and increased expression of home box genes like HOX B13 and PAX 2 genes [5]. Beyond these two trimesters, the fetal cells slowly acquire adult characteristics.

Perspectives on Fetal Growth and Development across Culture and Time

Fetal growth and development have been always an enigma that people over the ages have tried to

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unravel, with each having his own perspectives and answers.

The Egyptian texts show Akhnaton (Amenophis IV) praising the Sun God Aton as the creator of germ in a woman, maker of seed in men and giver of life. The ancient Egyptians professed that the soul entered the child at birth through the placenta.

A Hindu Scripture penned in Sanskrit embodying the development of the fetus has been traced to 1416 BC, and is known as the Garbha Upanishada.

Even Greek scholars made many contributions to this with books authored by the Father of Medicine, Hippocrates of Cos (Circa 460–377 BC) featuring the first recorded embryologic studies. Aristotle of Stagira (circa 384–322 BC), a Greek philosopher and scientist promoted the idea that the embryo developed from a formless mass, which he described as a "less fully concocted seed with a nutritive soul and all bodily parts" [6].

A Roman scientist, Claudius Galen (circa 130–201) described the development and nutrition of fetus in his book, *On the Formation of Fetus*.

The Jewish physician Samuel El Yehudi (second century AD) mentioned six stages of development.

Views in the medieval period hardly deviated from the theory of Aristotle. But with the dawn of the Renaissance, we find Leonardo da Vinci (1452–1519) making accurate sketches of pregnant uteri containing fetus (Fig. 5.1) and introducing the quantitative approach to embryology [7]. The revolution began with the publication of William Harvey's (1578–1657) book, *De Generatione Animalium*. He is said to be greatly influenced by one of his Professors Fabricius (1537–1619), the author of *De Formato Fetus* [8].

In 1759, Casper Friedrich Wolff proposed the layer concept, which formed the basis of the theory of Epigenesis [9].

These were followed by Genetic perspectives of fetal development with James Watson and Francis Crick deciphering the molecular structure of DNA in 1953 [10].

Rapid advances in Molecular Biology next started addressing issues related to temporal and spatial regulation of gene expression and the process of cellular commitment towards formation of various parts [11].

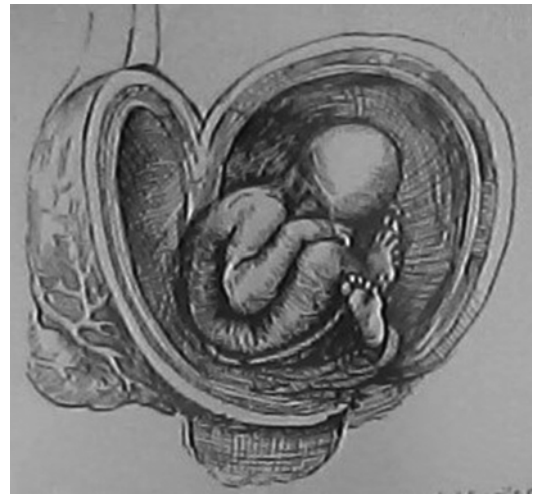

Fig. 5.1 Author's representation of Leonardo da Vinci's drawing made in the fifteenth century AD showing a fetus in utero

The Anatomical and Physiological Perspective of Fetal Development in the First Two Trimesters

For our convenience of understanding, the anatomical and physiological development of the fetus is discussed in periods of 4–5 weeks.

Nine to Twelve Weeks

At the beginning of the ninth week, the head constitutes approximately half the CRL of the fetus. Subsequently, growth in body length accelerates rapidly so that by the end of 12 weeks, the CRL has more than doubled. Although growth of the head slows down considerably by this time, it is still disproportionately large compared to the rest of the body [12].

At 9 weeks, the face is broad, the eyes are widely separated, the ears are low set, and the eyelids are fused. By the end of 12 weeks, primary ossification centers appear in the skeleton, especially in the cranium (skull) and long bones [13]. The legs are short and the thighs are relatively

small during the early ninth week. The upper limbs have almost reached their final relative lengths by the end of 12 weeks, but the lower limbs are still not so well developed and are slightly shorter than their final relative lengths.

The external genitalia of males and females are hard to distinguish until the end of the ninth week. It is by the 12th week that the mature fetal form is established. Intestinal coils are clearly visible in the proximal end of the umbilical cord until the middle of the tenth week. The intestines have returned to the abdomen by the 11th week.

At 9 weeks, the liver is the major site of erythropoiesis (formation of red blood cells) [14]. By the end of 12 weeks, the site of erythropoiesis shifts to the spleen. Urine formation begins between the 9th and 12th weeks, and urine is discharged through the urethra into the amniotic fluid. The fetus reabsorbs some amniotic fluid after swallowing it. It is through the placental membrane, that the fetal waste products are transferred to the maternal circulation (Fig. 5.2).

Thirteen to Sixteen Weeks

Rapid growth is observed during this period. By 16 weeks, the head is relatively small compared with that of the 12-week fetus and the lower limbs have lengthened. Limb movements, which first occur at the end of the embryonic period, become coordinated by the 14th week but are too slight to be felt by the mother [15].

Limb movements are visible during ultrasound examinations. Ossification of the fetal skeleton is active during this period, and the bones are clearly visible on ultrasound images by the beginning of the 16th week. Slow eye movements occur at 14 weeks. Scalp hair patterning is also determined during this period [16].

By 16 weeks, the ovaries are differentiated and contain primordial ovarian follicles that contain oogonia.

By 12–14 weeks, the genitalia of the fetuses can be recognized. The eyes face anteriorly rather than anterolaterally by 16 weeks. Besides, the external ears are close to their definitive position on the sides of the head.

Seventeen to Twenty Four Weeks

Growth slows down during this period, but the fetus still increases its CRL by approximately 50 mm [17]. Fetal movements—**quickening**—are commonly felt by the mother. The skin is now covered with a greasy, cheese-like material—***vernix caseosa***. It consists of a mixture of dead epidermal cells and a fatty substance (secretion) from the fetal sebaceous glands.

At 20 weeks, eyebrows and head hair are visible. The fetuses are usually completely covered with fine downy hair called **lanugo**, that helps to hold the *vernix caseosa* on the skin [18]. By 18 weeks, the fetal uterus is formed. The canalization of the vagina begins by this time with many primordial ovarian follicles containing oogonia being visible. By 20 weeks, the testes have begun to descend, but they are still located on the posterior abdominal wall, as are the ovaries in female fetuses.

Systemic Development

Respiratory System

- In the first two trimesters, lung development remains restricted to two of the four stages of maturation: **pseudoglandular** (6–16 weeks), and **canalicular** (16–26 weeks).
- In the beginning of the **pseudoglandular** period, the lungs only contain exocrine gland and when the fetus reaches 16 weeks, most of the important elements of the lung have been created except the part which involves gas exchange. Any fetus born during this period will not survive because no respiration is able to take place. By 20–22 weeks, type II pneumocytes begin to secrete pulmonary surfactant. Deficiency of surfactant results in respiratory distress syndrome (RDS) or hyaline membrane disease (HMD).
- The canalicular period refers to the period when there is growth of the lungs tree. The distal part of the lungs mature later than the proximal part. Owing to this the canalicular period overlaps with the pseudoglandular

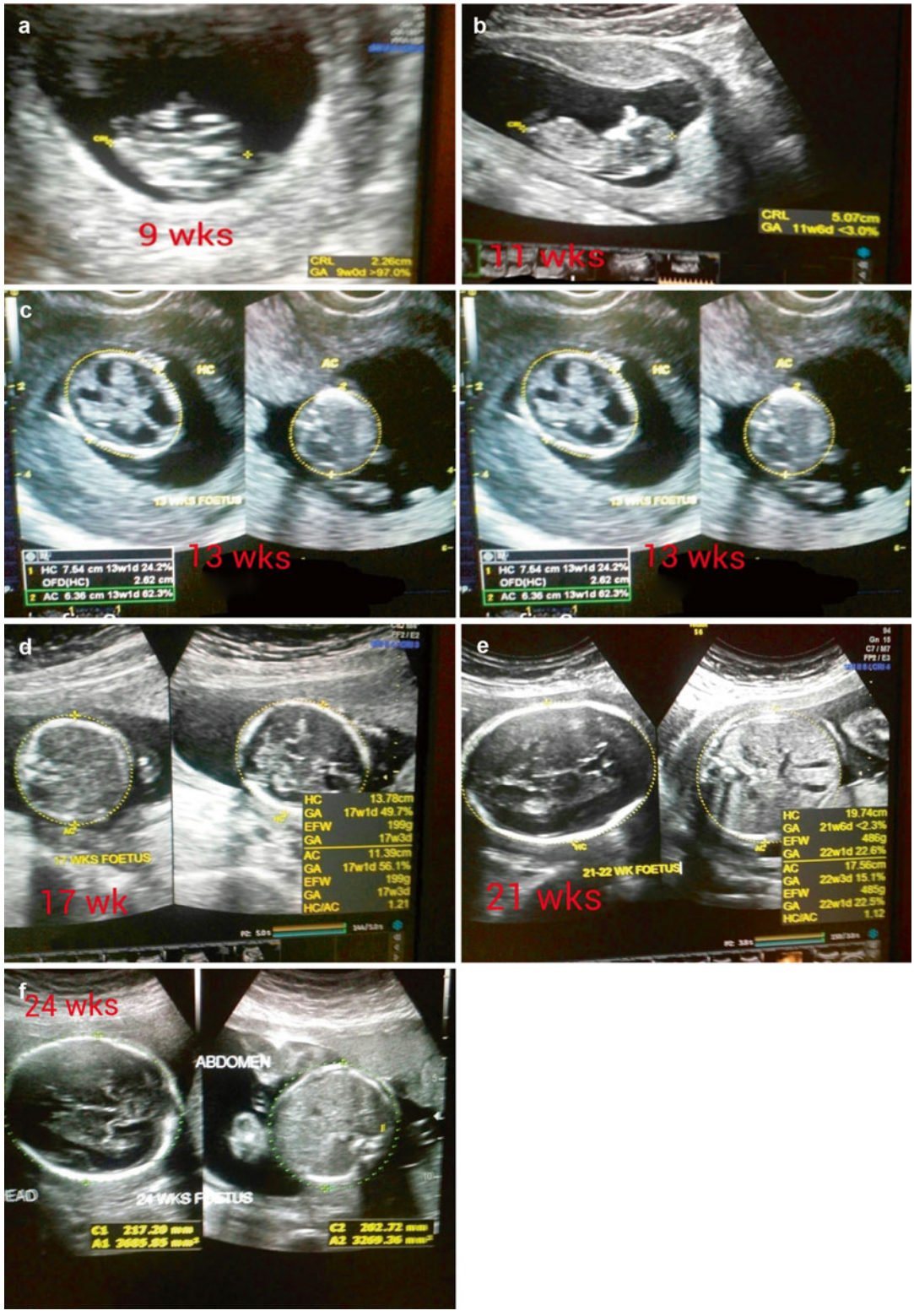

Fig. 5.2 (**a**) and (**b**) Sonographical images of fetuses of 9 weeks and 11 weeks of age, (**c**) and (**c** (i)) showing sonographical images of fetus of 13 weeks. (**d–f**) sonographical images of fetuses of 17, 21 weeks and 24th week of development

period. During this period, the lung tissue is highly vascularized and the lumen of the bronchi and terminal bronchioles are enlarged. Towards the end of the canalicular period, approximately between 24 and 26 weeks of gestation, it is possible for respiration to occur after the passages have completed development up to the primordial alveolar sacs and the lung tissues is also well vascularised [19].

Cardio-Vascular System

The cardiovascular system is the first major system to function in the embryo. The primordial heart and vascular system appear in the middle of the third week. This precocious cardiac development occurs because the rapidly growing embryo can no longer satisfy its nutritional and oxygen requirements by diffusion alone. Consequently, there is a need for an efficient method of acquiring oxygen and nutrients from the maternal blood and disposing of carbon dioxide and waste products. The cardiovascular system is derived mainly from:

- Splanchnic mesoderm, which forms the primordium of the heart
- Paraxial and lateral mesoderm near the otic placodes
- Pharyngeal mesoderm
- Neural crest cells from the region between the otic vesicles and the caudal limits of the third pair of somites.
- Endocardium formed from the Splanchnic mesoderm migrate and localize between the developing Myocardium above and the endoderm below. N- Cadherin may also be implicated in the cell sorting. The catenins are linked with these cadherin molecules and involved in the wnT signaling pathway in the development of the heart tube [20].

The fetal circulatory system operates very differently from that of the newborn, being organized in parallel circles [21]. Blood carrying the highest oxygen saturation goes to the fetal heart, the brain, the upper extremities while the other parts of the fetal body receive blood with lower oxygen saturation. Also fetal blood pO2 is much lower than maternal blood pO2 and hence fetal environment have been equated with Mount Everest in utero [22]. Despite the low pO2 in the fetal blood, the fetus is not exposed to hypoxia due to its adaptive responses and the hemoglobin concentration of fetal blood is about 50 % greater than that of the mother and interestingly the fetal hemoglobin can carry more oxygen at a low PCO2 than it can at high PCO2 and are alkaline in nature. Capacity of fetal blood to combine with oxygen increases, while maternal blood has diminished capacity to do so, thus enforcing the Double Bohr effect [23].

The first line of supervision over the blood circulation of the fetus are mediated by the carotid chemoreceptors, with the second line being hormones, Antidiuretic hormone (ADH), Angiotensin II, catecholamines and cortisol [24, 25].

Gastrointestinal System

The primitive gut that has already developed in the embryonic phase, starts peristaltic activities in the large intestine by 10 weeks and by 11 weeks in the small intestine [26]. Swallowing amniotic fluid also reflects fetal CNS maturity and contributes to somatic growth, development and maturation of the fetal Gastrointestinal System providing 10–15 % of nitrogen requirement in the normal fetus. Upper gastrointestinal tract obstruction in human fetuses are associated significantly with fetal growth restriction and amniotic fluid volume [27, 28]. Besides, fetal swallowing is influenced by hypoxia, hypotension and plasma osmololality changes as well [27]. The main feeding regulatory factors, neuropeptide Y (NPY) and Leptin are secreted in human fetuses as early as 16–18 weeks respectively [29, 30]. Leptin may be implicated in the development of the fetal gastrointestinal tract as well; this is suggested by the presence of Ob-Rb (functional receptor of Leptin) in the mucosa [31].

It is observed that by 13 weeks the intestine starts to absorb glucose and water [26]. Enzyme

activities increase after 14 weeks of gestation with the first traces of gastric acidity appearing in a 4 month old fetus [26].

Urinary System

From the beginning of the fourth week of the embryonic period the excretory organs starts to form pro-nephros, mesonephros and metanephros, with ultimately metanephros forming the definitive kidney. The nephrons are derived from the metanephric blastema [32]. The first nephrons appear in the kidney medulla, around 20–22 weeks of gestation. Failure in the maturation of the primitive kidneys can lead to abnormal development of the genital system, adrenal glands and lungs [33]. Studies have revealed that retinoids, metabolites of vitamin A, have a significant impact on the number of nephrons and this happens to be dose- dependent and are commonly associated with cigarette smoking, alcoholism and unbalanced diet. Ironically the congenital nephron deficit could be a missing link in understanding the etiopathogenesis of essential hypertension [34].

The fetus usually produces hypotonic urine and begins producing the anti-Diuretic hormone (ADH) by the 11th week of gestation. Aquaporin, special water channels appear by 12–15th week [35]. However the fetal kidney does not play a significant role in the acid base balance of the body [33]. It starts excreting urine from the third month of pregnancy [36].

Central Nervous System (CNS)

CNS develops from the embryonic ectoderm. After the early embryonic development characterized by its immobilization, the earliest neural activity originates from spinal motoneurons [37]. From early fetal period the first signs of supraspinal control on motor activity begins to be evident [38]. By the ninth week, the brainstem, the diencephalon and cerebral hemispheres are formed [39]. Facial movements, which are controlled by V and VII cranial nerve, appear around 10–11 weeks of gestation [38]. After the 12th week, the fetal movement become more variable in speed and amplitude [40]. While the midbrain maturation begins as late as the middle of the second trimester, that controls the eye movements [41]. The second trimester of pregnancy is characterized by organization of fetal movement patterns and increase in complexity of movements, with rest activity cycles becoming recognizable [42]. It is unknown whether and when the fetus begins to understand pain. Functional Thalamo-cortical connections are required for this awareness. It is supposedly formed between 22 and 26 weeks, and beyond this the fetus can probably feel pain since it is able to perceive the stimuli [43].

Genetic and Epigenetic Perspectives of Fetal Development

Understanding the epigenetic regulation of fetal development requires the integration of many fields of scientific inquiry including phylogeny, the evolution of placental anatomy, comparative genomics, and epigenomics. Pregnancies vary enormously in terms of timing of embryo implantation, placental anatomy, uterine shape, rates of fetal growth and development, neonatal body mass, number of offspring per pregnancy, and gestation length.

A growing body of evidence supports the notion that epigenetic changes such as DNA methylation and histone modifications, both involving chromatin remodeling, contribute to fetal metabolic programming. Research has revealed that impaired intrauterine growth and adult metabolic and cardiovascular disorders, including coronary heart disease, type 2 diabetes, and insulin (INS) resistance, are strongly associated. It was David Barker and coworkers, who followed-up a cohort of 499 men and women born in Preston (Lancashire, UK) during 1935–1943, and found correlation that adults having highest blood pressures were those who had been small babies with large placentas. Barker and Hales also explained that fetuses having an impaired supply of nutrients try to adapt by changing their physiology and metabolism, and

altering the sensitivity of tissues; this leads to an abnormal structures and functions in adult life. This led to the formulation of the "thrifty phenotype hypothesis".

A dynamic cross- talk between the fetal and maternal environment and modulation of gene expression was strongly hypothesised. Epigenetic modifications such as DNA methylation and covalent posttranslational histone modifications, which mediate phenomena such as genomic imprinting and chromatin remodeling, eventually emerged as a plausible molecular explanation of fetal metabolic programming [44].

Factors Influencing Fetal Growth and Development

The fetus requires various substrates for growth and production of energy and several other factors that govern fetal morphogenesis in varying proportion to the growth and development, the principal determinants being the fetal genotype and in utero environment.

Fetal Parameters

- **G**enetic factors contribute to fetal growth and development. Studies involving knock out genes or gene over-expression have revealed that both maternal and paternal influences are present during fetal development and are passed on to the developing fetus by spermatozoa or oogonia by a mechanism called imprinting [45]. It has been observed gynogenetic zygotes (two maternal genome copies) lead to under-developed extra-embryonic tissues but well developed embryos. Insulin like growth factor I (IGF I) and IGF II are two protein products of gene that specifically regulate the development of trophoblast cells, which form the placenta. Studies have shown that paternal IGF-II allele in mice model produce 60 % less fetal growth.
- **N**utritional factors that govern fetal growth and development, has the placenta providing a major role. During the first two trimesters the placenta is functional as a nutritive, respiratory, excretory and endocrine organ and from the very inception it is responsible for the diffusion of oxygen and nutrients from the maternal blood to the embryonic blood and passing the metabolic wastes in the reverse direction [46]. With increasing months of pregnancy the placental growth and thinning of its membrane diffusion layers, the placental diffusion rate gets a surge but exchange of oxygen is more related to the blood flow than the placental thickness [47]. The main ingredient of fetal diet is carbohydrate. The fetus has low capacity for gluconeogenesis as the necessary enzymes are inactive due to low fetal arterial pO2. The fetal insulin determines the glucose utilization by the fetus. Hormonal regulation of fetal growth differs from the post-natal regulation. ACTH and glucocorticoids stimulate the storage of glucose as glycogen [47]. Fetal growth hormone also has a small role in stimulating fetal growth as there is lack of functional GH receptors. But fetal insulin significantly stimulates fetal growth. It is known that pancreatic agenesis leads to fetal growth restriction whereas fetal hyperinsulinemea leads to fetal mass overgrowth [48]. IGFs are present in human fetal tissue extract after 12 weeks of gestation [49]. Its level in fetal and cord circulation directly correlates with fetal length and mass [2].

Maternal Parameters

- **P**arity, **m**aternal age and **m**ultiple pregnancy are also factors influencing fetal growth and development. Primiparous mothers have been found to bear smaller babies than multiparous mothers. Individuals of multiple births usually weigh considerably less than infants resulting from a single pregnancy. It is evident that the total metabolic requirements of two or more fetuses exceed the nutritional supply available from the placenta during the third trimester [50].
- **M**aternal Nutrition: There is growing evidence that maternal nutritional status can alter

the epigenetic state (stable alterations of gene expression through DNA methylation and histone modifications) of the fetal genome. This may provide a molecular mechanism for the impact of maternal nutrition on both fetal programming and genomic imprinting. Promoting optimal nutrition will not only ensure optimal fetal development, but will also reduce the risk of chronic diseases in adults [51].

- **C**igarette smoking is highly detrimental to fetal development as the mother inhales nicotine, lead, arsenic and carbon monoxide. These cause placental insufficiency. Smoking during pregnancy can cause low-birth weight, preterm delivery, and infant death. Smoking during pregnancy is estimated to account for 20–30 % of low-birth weight babies, up to 14 % of preterm deliveries, and about 10 % of all infant deaths according to the American Lung Association. Smoking is a well-established cause of IUGR (Intra uterine growth retardation) [52].
- **Al**cohol and Illicit Drugs: Fetal alcohol syndrome (FAS) or fetal alcohol syndrome is a pattern of physical and mental defects that can develop in a fetus in association with high levels of alcohol consumption during pregnancy. Alcohol crosses the placental barrier and can stunt fetal growth or weight, create distinctive facial stigmata, damage neurons and brain structures, which can result in intellectual disability and other psychological or behavioral problems, and also cause other physical damage [2, 53–55]. The main effect of FAS is permanent central nervous system damage, especially to the brain. Developing brain cells and structures can be malformed or have development interrupted by prenatal alcohol exposure; this can create an array of primary cognitive and functional disabilities (including poor memory, attention deficits, impulsive behavior, and poor cause-effect reasoning) as well as secondary disabilities.
- **I**mpaired utero-placental and Feto-placental Blood Flow: Maternal placental circulation may be reduced by conditions that decrease uterine blood flow (e.g., small chorionic vessels, severe maternal hypotension, and renal disease). Chronic reduction of uterine blood flow can cause fetal starvation resulting in IUGR. Placental dysfunction or defects can also cause IUGR. The net effect of these placental abnormalities is a reduction of the total area for exchange of nutrients between the fetal and maternal blood streams. It is very difficult to separate the effect of these placental changes from the effect of reduced maternal blood flow to the placenta. In some instances of chronic maternal disease, the maternal vascular changes in the uterus are primary and the placental defects are secondary [2].

Microchimerism

During the period of fetal development an interesting phenomena takes place called Microchimerism. It has only recently become apparent that naturally-acquired microchimerism is common in humans. The placenta was previously thought to barricade the mother and fetus thus preventing maternal rejection of the fetus. It is now known that during pregnancy some cells traffic from mother to fetus and from fetus to mother. Surprisingly, some of the mother's cells can be found in her adult offspring and some cells from the fetus can be found in the mother decades later. Microchimerism (Mc) refers to harboring a small number of cells or DNA from a genetically different individual [56].

Cytokine Response to Prenatal Infections during Fetal Development

Bacteria and viruses are potent activators of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which are key effectors of the peripheral immune response [57] and the main mediators of brain orchestrated sickness responses following infection and inflammation [58]. In the periphery these cytokines act in a cascade-

like fashion, which generally involves the sequential induction of TNF-α, IL-1β and IL-6 [59]. Although the cytokine-dependent inflammatory response is essential in resolving infection, abnormal changes in TNF-α, IL-1β and IL-6 levels during gestation, as a result of infection, may affect fetal brain development in utero [60]. Indeed, while cytokines have a key role in normal brain development [61], they are implicated in the death and dysfunction of neurons following injury or chronic disease in the adult brain [62], and are elevated in neurodevelopmental insults, such as hypoxia, also associated with an increased risk of schizophrenia [63]. Thus, cytokines are logical candidates to mediate the effects of maternal infection on the fetus; however, there have been contradictory reports of both increased and decreased expression of these mediators in the fetal brain following exposure of pregnant rats to bacterial LPS [64, 65]. Therefore, it remains unclear whether maternal LPS exposure during pregnancy results in altered cytokine expression in the fetal brain. Downstream consequences of systemic cytokine induction during pregnancy include changes in circulating levels of glucocorticoid [66, 67] and thyroid hormones [68, 69]. Maternal glucocorticoid [70, 71] and thyroid [72] hormones can cross the blood placental barrier and are essential for normal fetal neurodevelopment [73, 74]. However, in humans, exposure of the fetus to excess glucocorticoids can have detrimental effects on fetal brain development [73, 75].

Bacterial infection of the developing fetal lungs too begins with an inflammatory cascade resulting in cytokine injury and oxidative stress. For some pathogens like P. Falciparum, the mechanisms involve oxidative stress and apoptosis to disrupt placental and fetal growth. An in utero infection may also affect the long-term health of the infant; in many cases, a viral infection in utero increases the risk of developing type 1 diabetes in childhood. Understanding the varied mechanisms employed by these pathogens may enable therapies to attenuate changes in fetal development, decrease preterm birth, and improve survival.

Fetal Allograft

The feto-placental unit may be regarded as an allograft with respect to the mother, yet the maternal immune system does not reject it, giving rise to a major biologic enigma in nature. For nearly 50 years, the subject has been dominated by Medawar's 'fetal allograft analogy' hypothesis, in which the fetus is assumed to be a semi-allogeneic conceptus that evaded rejection [76].

The syncytiotrophoblast of the chorionic villi, although exposed to maternal immune cells within the blood sinusoids, lack major histocompatibility (MHC) antigens and hence no rejection responses are evoked. However, extravillous trophoblast (EVT) cells, which invade the uterine decidua and its vasculature (spiral arteries), express class I MHC antigens. These antigens include HLA-G, which, being nonpolymorphic (class Ib), is poorly recognizable by T lymphocytes as an alloantigen, as well as HLA-C, which, being polymorphic (class Ia), is recognizable by T cells. In addition to averting T cells, EVT cells must also shield themselves from potential attack by natural killer (NK) lymphocytes and injury inflicted by activation of complement [77].

The strategic location of HLA G in the placenta is believed to evade T-cell recognition owing to its nonpolymorphic nature, and recognition by the "killer-inhibitory receptors" on NK cells, thus turning off their killer function. Arguments against this hypothesis have been suggested by the identification of healthy individuals showing biallelic loss of HLA-G1, which goes on to sustain the thought that HLA-G is not essential for fetoplacental survival. Besides human EVT cells were found to be vulnerable to NK cell-mediated killing; and it was unable to explain the reason behind non rejection of HLA-C, a polymorphic antigen, also expressed by EVT cells.

A probable explanation could be that both HLA-G and HLA-C were shown to have the unique ability to resist human cytomegalovirus-mediated MHC class I degradation, with the probability of having selective location of these two antigens at the fetomaternal interface which may help to withstand viral assault.

- Immunoprotection is provided locally by certain immunosuppressor molecules, such as prostaglandin E_2, transforming growth factor (TGF)-β and interleukin-10. Decidua-derived prostaglandin E_2 was shown to block activation of maternal T cells as well as NK cells in situ. Indeed, immunoregulatory function of decidual cells is consistent with their genealogy. It was shown that uterine endometrial stromal cells, which differentiate into decidual cells during pregnancy, are derived from progenitor (stem) cells that migrate from hemopoietic organs such as the fetal liver and the bone marrow during ontogeny.
- Transient tolerance of maternal T-cell to the fetal MHC antigens may be a backup mechanism for placental immunoprotection. A similar B-cell tolerance has also been observed.
- A trafficking of activated maternal leukocytes into the placenta or the fetus is prevented by deletion of these cells triggered by apoptosis-inducing ligands present on the trophoblast.
- Based on genetic manipulation in mice, it was shown that the presence of complement regulatory proteins (Crry in the mouse, membrane cofactor protein or CD46 in the human) [78], which can block activation of the third component of complement (C3) in the complement cascade, protects the placenta from complement-mediated destruction, which may happen otherwise because of residual C3 activation remaining after defending against pathogens. Crry gene knockout mice died in utero because of complement-mediated placental damage, which could be averted by additional knockout of the C3 gene.
- Experiments in mice revealed that the presence of the enzyme indoleamine 2,3-deoxygenase in trophoblastic cells was critical for immunoprotection of the allogeneic conceptus by suppressing T cell-driven local inflammatory responses including complement activation [79].

Another hypothesis is put forward to account for the non-rejection of the fetal allograft, based on shared surface-repellent molecules (S.R.M.s) which prevent close apposition of maternal and fetal immunocompetent cells. This hypothesis is shown to be compatible with the major theoretical requirements of the immune system. Thus it provides possible explanations for the self/not-self recognition process, tolerance to organ transplants, the development of autoimmune disease, and high and low zone tolerance. The recognition of self is important with reference to cellular antigens, but lesser less so for humoral antigens [80].

The survival of the fetal allograft also depends on a fine well coordinated systemic and uterine support by the reproductive hormones Progesterone (PR). Estrogen (ER), Human chorionic gonadotropin (HCG), prolactin (PRL), and others. These hormones and their cytokine nexus are essential for maintaining the pregnancy specific immunomodulation, including its up and down regulations.

Diagnostic Parameters

Ultrasonography

Ultrasonographical examination of the fetus became integrated into the prenatal care soon after its introduction in the 1950s, because of its wide availability, low cost, and lack of known adverse effects. The chorionic sac and its contents may be visualized by ultrasonography during the embryonic and fetal periods. The benefits of antenatal ultrasound examination can proceed from assessment in the first trimester. Determination of risk assessment of aneuploidy in singleton and multiple gestations, fetal viability, accurate gestational age, multiple gestations and chorioicity and amnionicity and detection of fetal anomalies have been described with first trimester ultrasonography [81].

The benefit burden calculus of routine antenatal ultrasonography support its use and fulfillment of ethical principles of beneficence and respect for patient's autonomy [82].

Ultrasound scans give accurate measurements of the biparietal diameter of the fetal cranium (skull), from which close estimates of fetal age and length can be made. Despite difficulty of accurate diagnosis, detection rate of fatal skeletal abnormality with ultrasonography is as high as 94–96 %. With the advancement in technology of

three-dimensional ultrasonography, abnormality in face, relative proportion of limbs, hands and feet are observed with more accuracy [83].

Diagnostic Amniocentesis

Amniocentesis is a diagnostic test that may be recommended following an abnormal triple test result. This is a common invasive prenatal diagnostic procedure, usually performed between 15 and 18 weeks gestation. Amniotic fluid is sampled by inserting a 22-gauge needle through the mother's anterior abdominal and uterine walls into the amniotic cavity by piercing the chorion and amnion. Because there is relatively little amniotic fluid before the 14th week, amniocentesis is difficult to perform before this time. The amniotic fluid volume is approximately 200 ml, and 15–20 ml can be safely withdrawn [84]. Amniocentesis detects chromosome abnormalities, neural tube defects and genetic disorders. Down syndrome or Trisomy 21 is the most common chromosome abnormality. Genetic disorders include disorders like cystic fibrosis. The most common neural tube defect is spina bifida. Amniocentesis is occasionally used late in pregnancy to assess fetal lung maturity [84, 85].

Alpha Feto Protein Assay

Alpha-fetoprotein (AFP) is a glycoprotein that is synthesized in the fetal liver, umbilical vesicle, and gut. AFP is found in high concentration in fetal serum, peaking 14 weeks after the LNMP. Small amounts of AFP normally enter the amniotic fluid. This protein is produced early in gestation by the fetal yolk sac and then later in the liver and gastrointestinal tract. The true function of AFP is unknown. We do know that this protein's level increases and decreases during certain weeks of pregnancy which is why accurate pregnancy dating is crucial.

The AFP test is measuring high and low levels of alpha-fetoprotein. The results are combined with the mother's age and ethnicity in order to assess probabilities of potential genetic disorders. Amniotic fluid AFP concentration is measured by immunoassay, and, when used with ultrasonographic scanning, approximately 99 % of fetuses with these severe defects can be diagnosed prenatally [85].

Characterization of First Trimester Fetal Erythroblasts for Non-invasive Prenatal Diagnosis

Isolation of fetal nucleated red blood cells (NRBC) from maternal blood would allow non-invasive prenatal diagnosis of chromosomal and monogenic disorders without the inherent risks of invasive procedures [86].

Components of this technique include enrichment, identification and molecular genetic diagnosis. One study showed that primitive erythroblasts were the predominant cell type until 12 weeks gestation, after which time their numbers declined steeply; 100 % were ϵ-globin-positive versus <0.06 % definitive erythroblasts. Buoyant densities of first trimester fetal erythroblasts ranged from 1.077 to 1.130 g/ml, and optimal recoveries were obtained with Percoll 1118. Although primitive erythroblasts carried a negative surface charge and were resistant to NH_4Cl lysis, these properties had only a limited role in fetal cell enrichment. Immunophenotyping showed that primitive, like definitive, erythroblasts were GPA+, CD47+, CD45– and CD35–, whereas CD71 expression was weak/undetectable on primitive erythroblasts but strongly positive on 100 % of definitive erythroblasts; primitive erythroblasts were also CD36– whereas definitive erythroblasts were CD36+. Using chromosomal fluorescence in-situ hybridization (cFISH) or polymerase chain reaction (PCR) [87].

Spectrophotometric Studies

Examination of amniotic fluid by this method may be used for assessing the degree of erythroblastosis fetalis, also called hemolytic disease of the newborn. This disease results from destruction of fetal red blood cells by maternal antibodies. In 1961, Liley demonstrated the prognostic value of amniotic fluid spectrophotometry to

identify these infants and then showed that intrauterine transfusions could prevent fetal deaths [88].

Chorionic Villus Sampling

Chorionic *villous* sampling (CVS) is a form of prenatal diagnosis to determine chromosomal or genetic disorders in the fetus. It entails sampling of the chorionic villus (placental tissue) and testing it for chromosomal abnormalities, usually with FISH or PCR. CVS usually takes place at 10–12 weeks' gestation, earlier than amniocentesis or percutaneous umbilical cord blood sampling. It is the preferred technique before 15 weeks [89, 90]. CVS was performed for the first time by Italian biologist Giuseppe Simoni, scientific director of Biocell Center, in 1983.

Conclusion

The fetus in its struggle for survival in the first two trimesters braves the environmental mileau that it is exposed to and adapts and modifies to realize its developmental potential determined by genetic and epigenetic factors. These two trimesters provide the fetus with unique protective abilities as the fetal cellular characteristics are quite divergent from its postnatal characteristics, being many times more sensitive, with the ability to heal without scar formation and fetal stem cells having much higher proliferative potential as well as differentiation capacity as the fetal tissue extracted in this period are able to regenerate diseased and damaged tissues with minimal GVHD. The fetal growth and development in the first two trimesters masterminds the health outcome of the adult and are hence of paramount importance.

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Part II

Structural and Functional Fetal Development up to Second Trimester

Anthropometric Measurement of the Human Fetus

6

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Anthropometric Measurement of Human Fetuses

The present chapter reports the findings of a study conducted at the Institute of Post Graduate Medical Education and Research, Calcutta, by a group of researchers led by Prof KL Mukherjee, who headed the Department of Biochemistry. The study was conducted from 1977 to 1987. The foetuses were collected from SSKM Hospital, Calcutta, with the informed consent of mothers who had undergone medical termination of pregnancy, which is legal under India's MTP law under certain circumstances. For the sake of convenience the fetuses have been divided into six groups: – A, B, C, D, E and F according to a difference in the gestation period of 4 weeks. The gestational age was ascertained form the date of the last menstrual period.

Table 6.1 shows the grouping and anthropometric measurement of human fetuses in this series.

Group A contained 90 fetuses of 9–12 week of gestation. Their weights varied form 1–14 g, the C.R. lengths form 22–67 mm, C.H. lengths form 30–96 mm and H.C. form 34–82 mm.

Group B contained 337 fetuses of 13–16 weeks of gestation. Their weights varied form 15–105 g, the C.R. lengths from 62 to 113 mm, C.H. lengths form 72 to 185 mm, and H.C. lengths form 58–145 mm.

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Table 6.1 Grouping of fetuses and anthropometric measurements

Group	Gestation period (wks)	Range of body weight (gms)	No. of cases	C.R. (mm)	C.H. (mm)	H.C. (mm)
A	(9–12)	(1–14)	90	22–67	30–96	34–82
B	(13–16)	(15–105)	337	62–113	72–185	58–145
C	(17–20)	(106–310)	425	107–228	107–330	130–248
D	(21–24)	(311–640)	531	140–220	169–372	160–235
E	(25–28)	(641–1,071)	10	193–247	303–336	198–270
F	(29–32)	(1,072–1,520)	2	250	400	280
G	(33–36)	(1,521–2,196)	1	280	418	316

CR Crown Rump
CH Crown Heel
HC Head Circumference

Group C contained 425 fetuses of 17–20 weeks of gestation. Their weights varied form 106–310 g, the C.R. lengths from 107 to 228 mm, C.H. lengths form 117–330 mm, and H.C. lengths form 130–248 mm.

Group D contained 531 fetuses of 21–24 weeks of gestation. Their weights varied form 331–640 g, the C.R. lengths from 140 to 220 mm, C.H. lengths form 169–372 mm, and H.C. lengths form 160–235 mm; some still born fetuses of longer gestation periods were available for these measurements. They include E,F and G.

Group E contained ten fetuses of 25–28 weeks of gestation. Their weights varied form 641–1,071 g, the C.R. lengths from 193 to 247 mm, C.H. lengths form 303–336 mm, and H.C. lengths form 198–270 mm.

Group F and G contained only three, whose gestation period were 29–32 and 33–36 weeks, C.R., C.H. and H.C. lengths in group F, 250 mm, 400 mm and 280 mm respectively. In group G they were 280, 418 and 316 mm respectively.

Since much of the body weight consists of the skeleton, it is apparent that the overall anthropometric measurements bear curvilinear relationship to the body weight (Fig. 6.1).

Fetuses of group E to group G were all still born fetuses.

Table 6.2 and Fig. 6.2 shows the relation between the body weight and the corresponding crown rump lengths. There is no straight line relationship between the length and the body weight of fetuses. The slope of the figure indicates that the rate of length (mm) change per unit mass (gm) decreases with time. Figure 6.1 shows that the body weight increases with progress of gestation and rate of increase of body weight rises with progress of gestation.

The question here is how crown-rump length (CR), crown-heel length (CH), head circumference (HC) grow in relation to body weight of human fetuses at different periods of gestation; this issue has been the subject of great concern to bio-statisticians over decades. Studies on the variation of these vital biological parameters during human fetal growth are based mainly on fitting of linear curves [1–7]. In our analysis (Figs. 6.3, 6.4, and 6.5) we have fitted three different curves for the purpose of analysis of trends in growth of CR, CH and HC corresponding to different body weights of human fetuses from 9 to 28 weeks of gestation.

We related the various anthropometric parameters to the fetal body weight. The body weight itself is a function of growth in respect to gestational age. There will be variations in the body weights of fetuses of a given gestational age. Thus a fetus of 16 weeks of gestation may weigh from 90 to 110 g. This amount of variation applies to all our anthropometric measurements.

There is an intrinsic growth rate of a normal fetus with respect to time in a given species. We have no idea about the factor controlling this rate of growth. We cover up our ignorance by saying that the rate of growth of a particular human fetus depends upon its intrinsic growth potential, inherent in its genomic complex which is modified by the environment, provided locally by the mother's uterine complex. It we assume that the local environment of a normal mother is optimal the individual growth of the fetus is a function of the genetic potential. Furthermore, the fetus is a

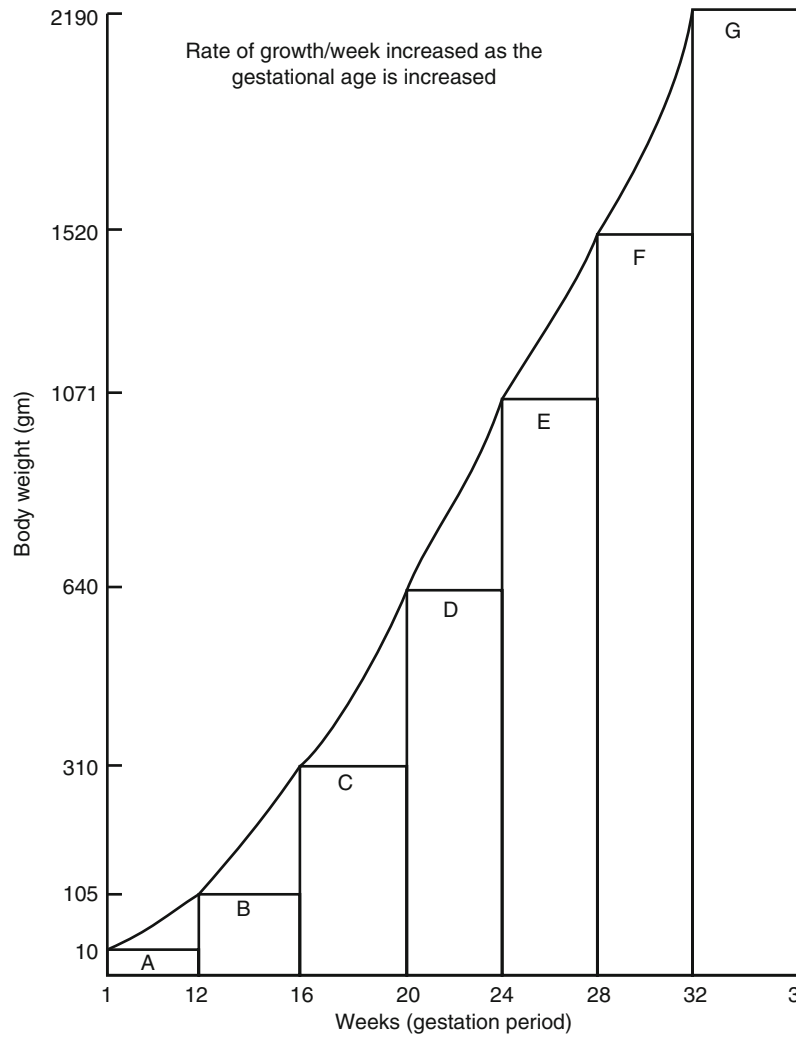

Fig. 6.1 Relationship of fetal body weight to overall anthropometric measurements

Table 6.2 Grouping of fetuses and weight length relationship

Group	No. of fetuses	Week	Weight (gm)	Length (mm) (crown rump)	Length/weight
A	15	8–12	1.5–14.5	25–53	4.6
B	25	13–16	15.6–103	62–109	1.1
C	36	17–20	115–295	110–157	0.90
D	21	21–24	330–660	163–211	0.37
E	14	25–28	715–1,025	203–252	0.20
F	3	29–32	1,055–1,650	230–277	0.17

composite of the growth of the organs it is constituted of. Growth of the individual organs is conditioned again by the genetic potential and the environment provided by the mother and a third component, the fetus itself. With these limitations in mind, we approach the data on the relationship of the various anthropometric measurements to fetal growth (which is the body weight in our case). Such relationships have been determined by a number of workers including ours [8–14]. Older data were based on the Carnegle Institute Collection. Streeter et.al. [15] and Schults et.al.

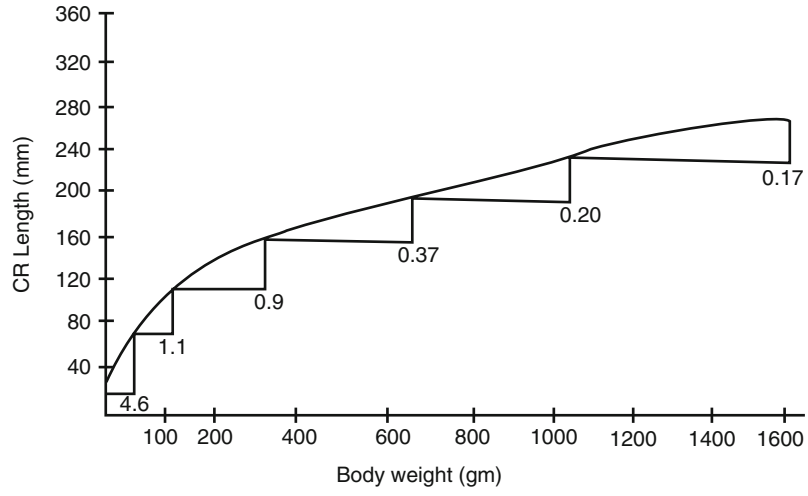

Fig. 6.2 Relationship of fetal body weight to crown-rump length

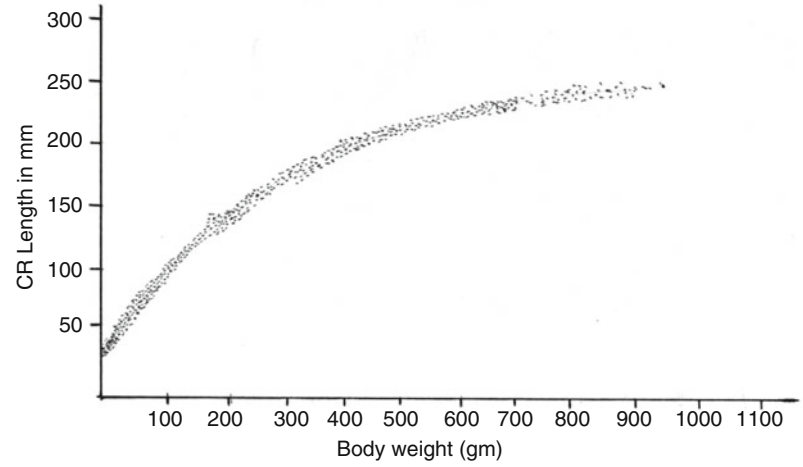

Fig. 6.3 Trend in growth in crown-rump length in relation to developing body weight of foetuses (9–28 weeks)

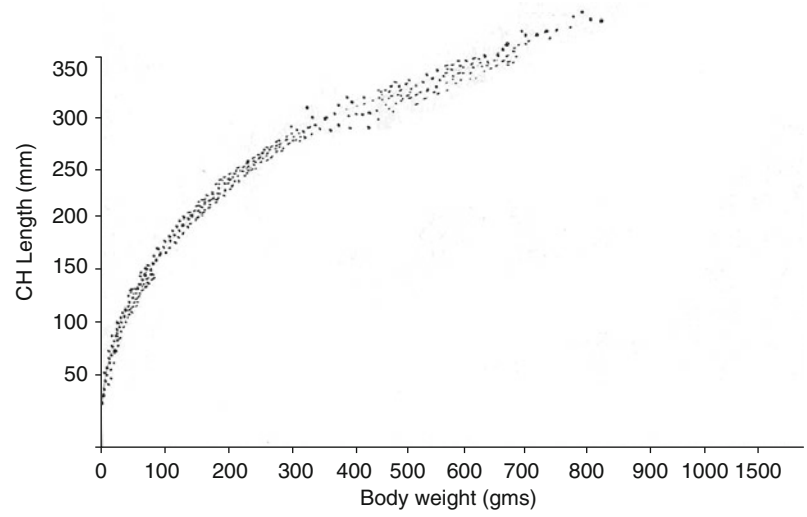

Fig. 6.4 Trend in growth in crown-head in relation to developing body weight of foetuses (9–28 weeks)

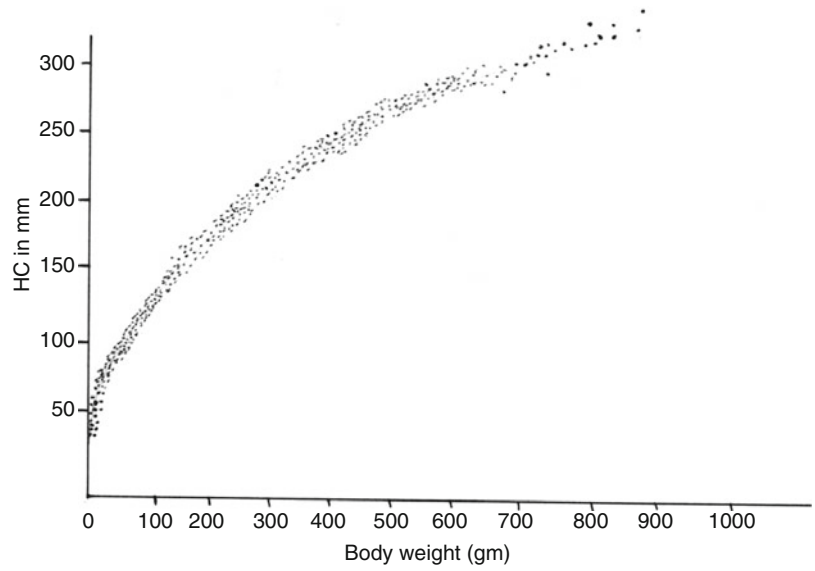

Fig. 6.5 Trend in growth in head circumference in relation to developing body weight of foetuses (9–28 weeks)

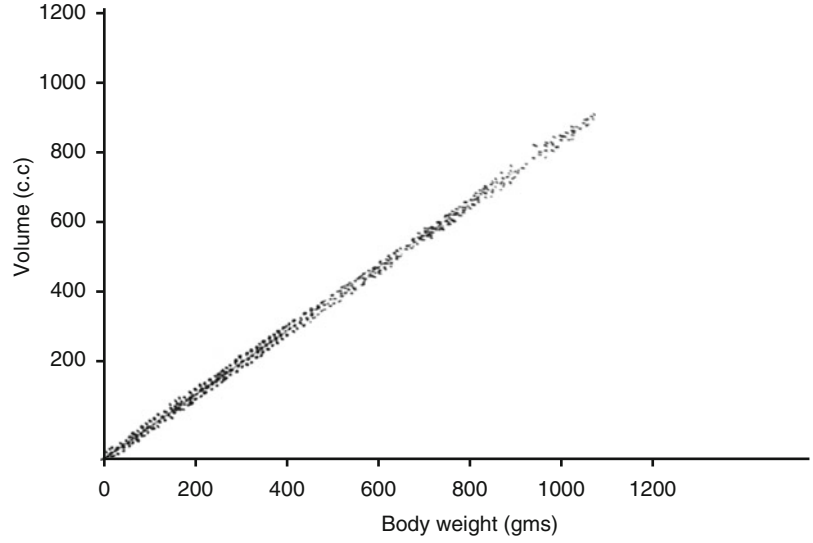

Fig. 6.6 Relationship between volume and body weight

[16, 17] studied the dimensions in fetuses obtained from spontaneous abortions or pathological pregnancies, on mainly formal and fixed specimens. Figure 6.6 shows the volume of fetuses bears a relationship to the body weight which almost passes through the origin of X and Y axis.

Anthropometry of Individual Organs

The growth of an organ from inception to a definitive functional stage is an integrated function of the whole organism. The related information of such growth is inbuilt in the nucleic acids of the cells of the organ. Such growth is probably independent of the development of the organism as a whole, except in so far as it is a subject to hormonal regulation. However, when it starts functioning, the functioning may be important for the development of the whole organism and thus will be related to the overall growth. As noted before organogenesis and throughout fetal life there is a characteristic growth rate of each individual organ of the fetus. In most organs the process is continued after birth.

Table 6.3 Relationship between the body weight and the weight of liver of human fetuses

Group	Period of gestation (weeks)	Number of cases	Body weight mean ± S.D (gm)	Liver weight mean ± S.D (gm)	Liver weight per 100 g body weight (gm)
A	9–12	74	9 ± 3	0.41 ± 0.20	4.60
B	13–16	208	50 ± 25	2.07 ± 1.04	4.25
C	17–20	192	187 ± 56	7.70 ± 2.36	4.13
D	21–24	68	420 ± 80	16.70 ± 3.35	3.95
E	25–28	27	738 ± 116	30.10 ± 4.38	4.10

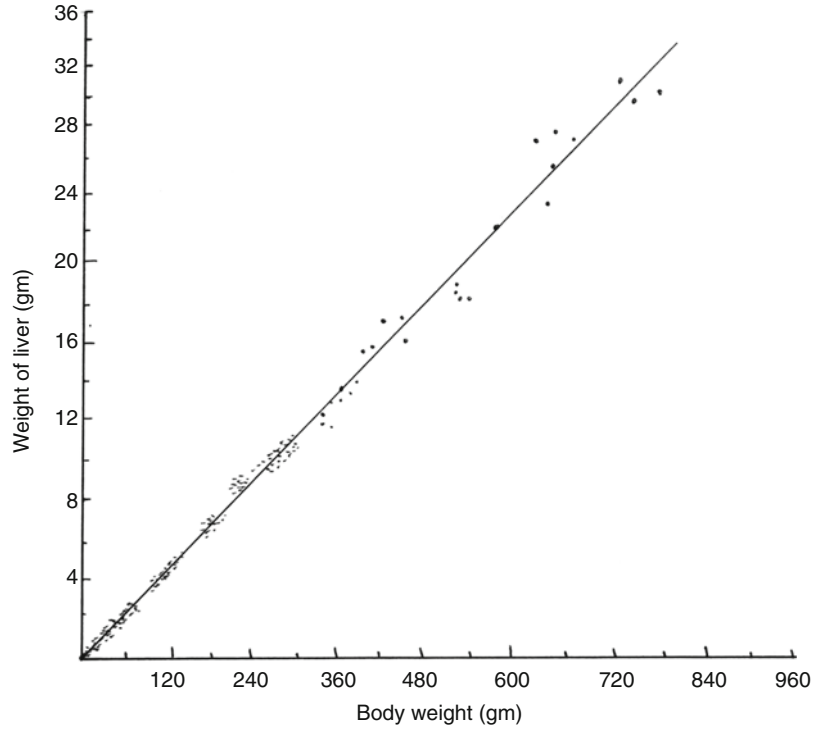

Fig. 6.7 Relationship of liver weight to body weight

As the fetus grows during gestation, its organs also grow in progression to the body weight. Regulation of the growth process is complex and not completely understood, while the genetic potentiality of the different endocrine organs related to growth and various unknown parameter mediate the growth regulatory process. It is important to determine some of these parameters.

Growth of the Liver

The growth in mass of the human fetal liver was compared to the overall body weight of the fetus. The liver at 8–12 weeks of gestation is relatively a bigger organ with respect to body weight, forming 4.5–5.5 % of body weight. It protrudes through the abdominal wall. Thereafter from 13 to 32 weeks of gestation it forms 3.4–4.0 % of body weight. As a result the liver weight forms a more or less constant proportion of body weight. Results are shown in Table 6.3 and Fig. 6.7. They show that the liver weight is directly proportional to body weight.

Growth of the Lung

The lung is a respiratory organ, but it is not so in fetal life, although the fetal lungs are known to expand and contract in the last stages of development. Therefore, whatever growth pattern is

observed in the fetal lung, it will be an intrinsic pattern of the organ.

The total weight of the right and left lungs and both lungs together are determined at different periods of gestation and expressed as a function of the total body weight. Results are shown in Table 6.4 and Fig. 6.8. There was a variation in individual fetuses; the weight of right and left lung and total weight of the lungs increased with the total weight of the fetus (Fig. 6.8). When the weight of the lung was expressed as gm/Kg of body weight (Table 6.4) and plotted against the body weight, we get somewhat irregular plot as shown in Fig. 6.9. Initially, that is, upto a body weight 350 g, there was a rather sharp decline in the rate of growth of the lungs. The decline was maintained upto a body weight of 850 g after which the rate of growth was uniformly proportional to the total body weight.

Growth of the Brain

The brain, for our studies, included the central nervous system upto the medulla at the level of the second cervical vertebra. The whole mass of tissue was dissected out, the cerebrospinal fluid was drained by decantation and the weight of the brain was determined. The smallest fetus we could examine weighed 1.5 g and thus belonged to a gestation period of 8.5 weeks. The brain at this time had already assumed the appearance of a human brain; i.e., the primary divisions and flexures had already occurred previously and the prosen, messen, and rhomben cephalon had already given rise to the constituent derivatives of the brain like rhinencephalon, corporastraita, cerebral cortex, thalamus and epi and hypothalamus, col-

Table 6.4 Weight of fetal lung in different gestation period

Gestational age (weeks)	Body wt. (gm)	Number of fetuses	Lung wt. (M ± SD) gm/Kg of body wt.		
			Right lung	Left lung	Total lung
12–16	14–105	10	19 ± 2.5	16 ± 2.1	35 ± 1.0
16–20	105–310	9	16 ± 1.6	13 ± 1.6	30 ± 3.4
20–24	310–640	18	15 ± 2.0	12 ± 2.1	27 ± 2.3
24–28	640–1,080	11	12 ± 1.2	10 ± 0.8	22 ± 2.0
28–32	1,080–1,650	10	*12* ± 2	*10* ± 1.4	*22* ± 2.8

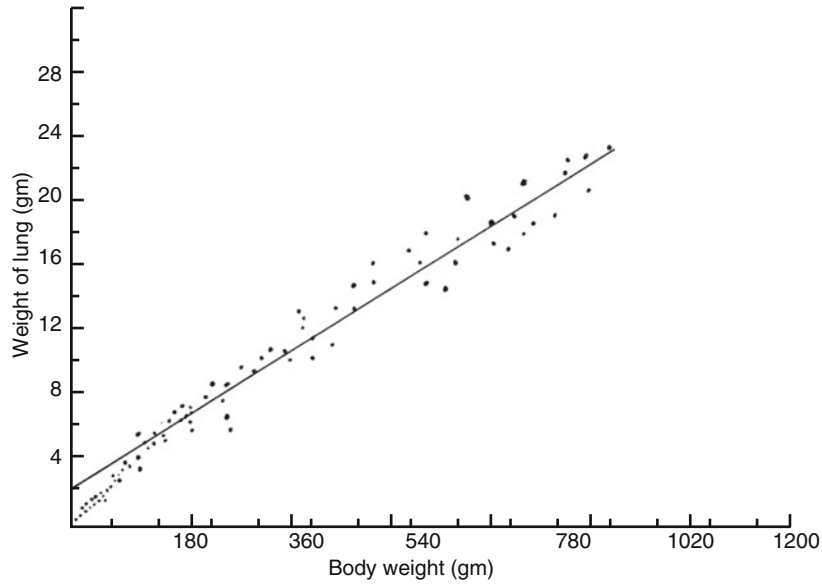

Fig. 6.8 Relationship of weight of lungs to body weight

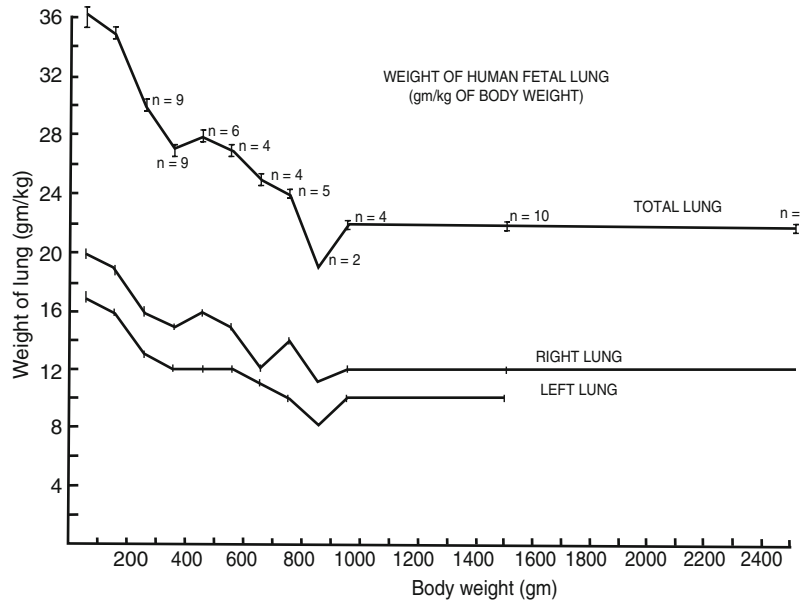

Fig. 6.9 Relationship of weight of lungs to body weight

Table 6.5 Brain weight g/Kg. of body weight

Body wt.	No. of samples	Mean ± S.D
0–100	26	172 ± 28
100–200	14	165 ± 26
200–300	12	160 ± 17
300–400	5	153 ± 23
400–500	3	165 ± 15
500–600	2	130±
600–700	3	157 ± 12
700–800	3	191 ± 14
800–900	2	160±
900–1,000	3	172 ± 16
1,000–2,000	5	157 ± 15

liculi, tegmentum, crurcerebri, cerebellum, pons and medulla, albeit to a relatively less differentiated extent. From this time onwards the growth of the brain was proportional to the weight of the fetus. The brain weight per Kg of body weight is shown in Table 6.5 and Fig. 6.10. It formed 16–18 % of the body weight. There were occasional fetuses whose brains weighed 20 % of the body weight and some brains were 12–13 % of the body weight. But by and large, the brains weighed 16–18 % of the body weight. Figure 6.11 shows that brain weight is directly proportional to the body weight.

Why the variations of weight are related to the large capacity of the brain is not known. Thus, why the brain of Stephen Hawking may belong to the first groups of 16–18 % of the body weight, while the brains of Tom, Dick and Harry may belong to the second group of 12–13 % of body weight, is not known.

Growth of the Kidney and the Adrenal Gland

Table 6.6 and Fig. 6.12 shows the total weights of the adrenal glands and the kidneys in 12 human fetuses arranged according to body weight. In early periods of gestation, the adrenal glands out weighed even the metanephric kidneys. It was comparatively a bigger organ. After the tenth week of fetal life, the kidneys began to grow in mass at a faster rate than the adrenal glands so that the adrenals and the kidneys weighed more or less the same upto the 12th week. Thereafter, the kidneys weighed more than the adrenals. But even so, the adrenal gland is a comparatively bigger organ in the fetus than in an adult.

Table 6.7 shows the adrenal weights of 90 human fetuses of different gestation periods. Figure 6.12 shows the results graphically where the weights of the adrenal glands are plotted against gestation age. There is a progressive

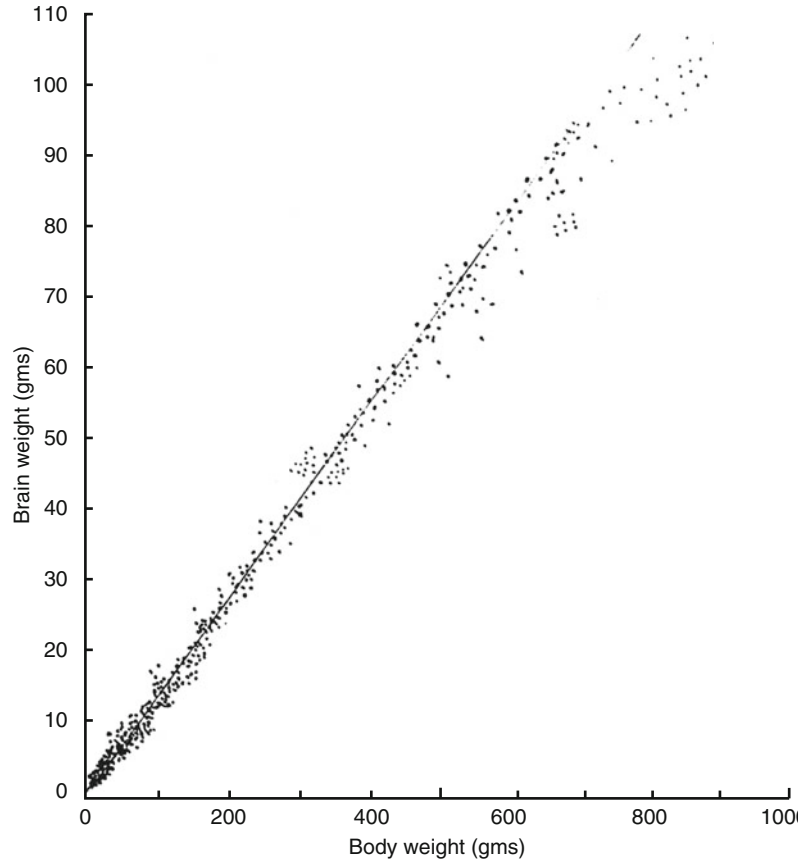

Fig. 6.10 Brain weight per Kg body weight

increase in the weights of the adrenal glands as the fetus increase in weight. The rate of increase, however, varies at different gestational periods, and is not uniform all throughout. Figure 6.13 shows the weights of adrenal glands expressed as mg. per gm of body weight against gestational age. There is a progressive decrease in the relative weight of the adrenal glands as the fetus advances in age. The rate of decreases is not uniform and greatest after birth.

Growth of the Human Fetal Testes

The growth and development of the testes in human fetuses are not uniform throughout gestation. The right and left testes were dissected individually, weighed and expressed as mg tissue weight. Results are shown in Table 6.8. The right testes were, as a rule, 4–10 % heavier than the left with few exceptions. There was a gradual increase in the weight of the testes (combined weights) as the gestation increased. The individual weights of the combined testes in mg were plotted against the weight of the fetus in gms. Results are shown in Fig. 6.14. The growth was not uniform; initially there was a proportional growth which soon flattened to increase with different slopes again and again throughout the whole length of the gestation. This is in marked contrast with the growth of an organ in mg per 100 g of body weight and results are plotted in a graphic form, (Fig. 6.15). There is a steep decline in the early gestation periods from about 200 mg per 100 g of body weight to about 60 mg per 100 g when the fetus weighted from 1.5 g to about 20 g. Later on in gestation, the decline is gradual. We could obtain data only upto a body weight of about 1,560 g when the testes weighed about 20 mg per 100 g of body weight. The decline was not uniformly maintained throughout the gestation period.

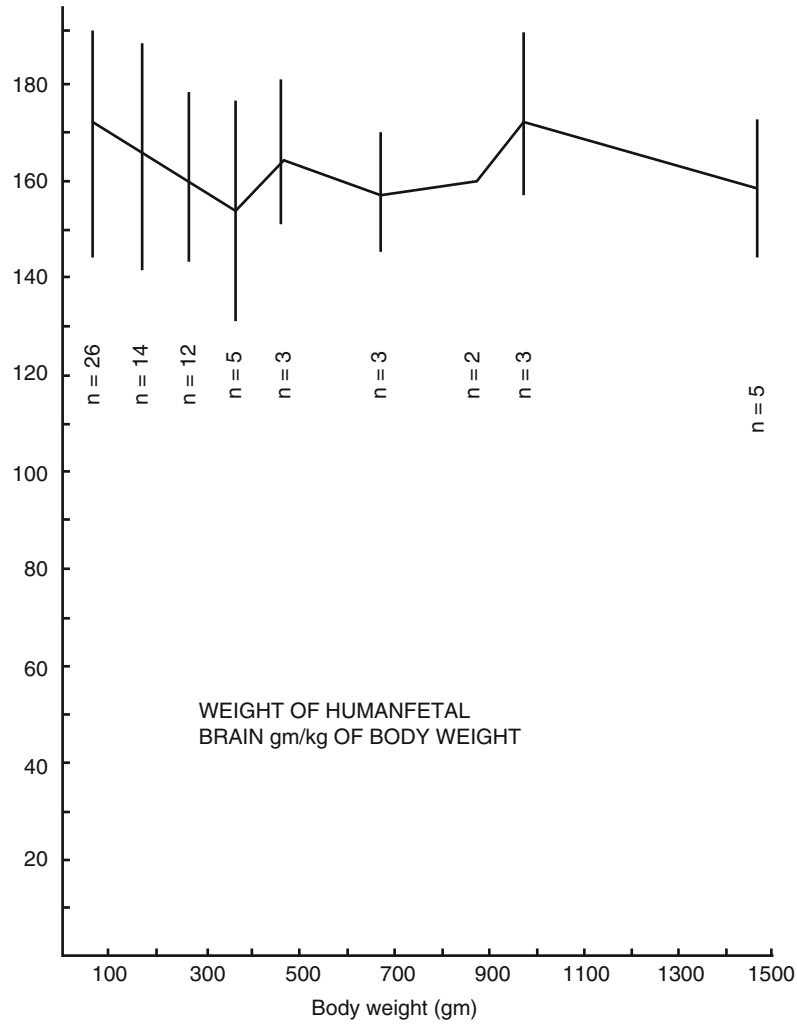

Fig. 6.11 Proportion of brain weight to body weight

Table 6.6 Relationship between the total weight adrenal glands and kidneys with the increase of body weight

Body weight (gm)	Approx. gestation period (weeks)	Total weight of kidneys (gm)	Total weight of adrenal glands (gm)
6.578	10	0.0145	0.0266
7.0702	10	0.0235	0.0320
11.1834	11	0.0391	0.0264
13.2	11.5	0.0666	0.0511
14	12	0.1141	0.1010
14.4755	12	0.0711	0.0535
102	16	0.6011	0.2731
280	19.4	2.3626	0.9658
457	21.6	2.8350	1.3713
735	24.6	4.6049	2.3364
1,010	28	8.609	2.4395
1,650	32	13.350	4.1457

Growth of the Human Fetal Ovaries

The ovaries were dissected individually from each side and immediately weighed. Results are shown in Table 6.9 and Fig. 6.16. There was a progressive increase of the weight of the ovary with increase in body weight. In most instances fetuses of identical body weights had almost identical ovarian weights. But occasionally, within each group there were a few fetuses, whose ovarian weights did not have the expected ovarian weights.

The individual weights of the combined ovaries in mg were plotted against the weight of the fetuses in gms. Results are shown in Fig. 6.17. Like the testes, here also the growth was not uniform.

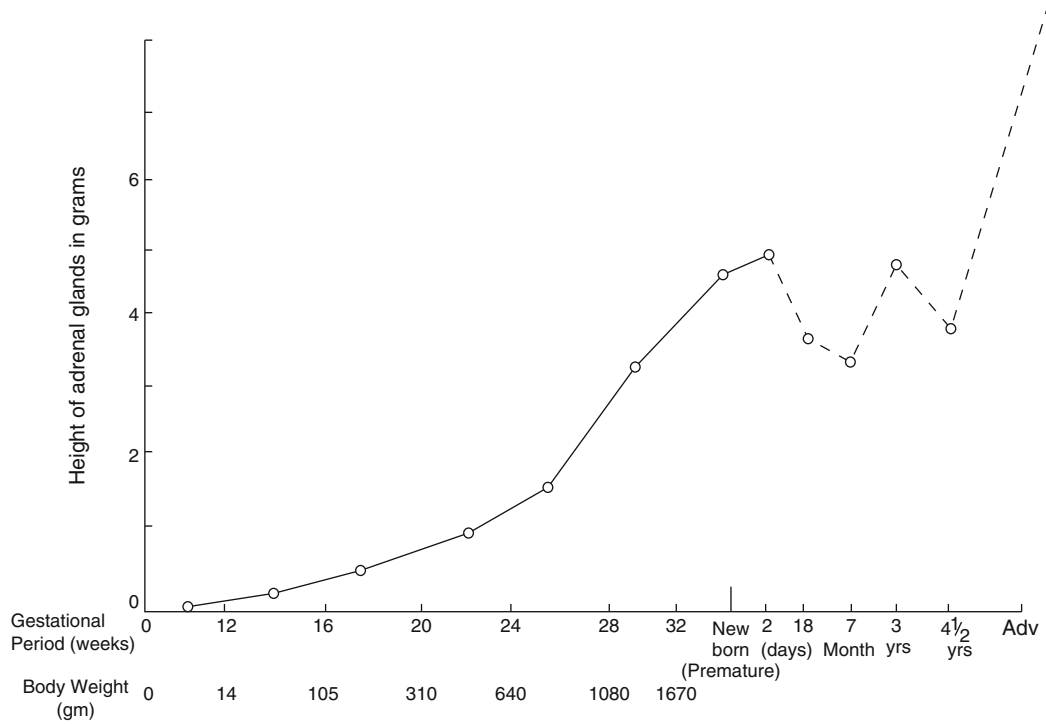

Fig. 6.12 Relation of weight of adrenal glands to gestational age

Table 6.7 Weight of the adrenal glands at different periods of human fetal life and upto adult

Gestational period (weeks)	No. of cases	Total weight of the adrenal
Below 12 weeks	7	0.0451 ± 0.0277
12–16 weeks	23	0.1623 ± 0.0097
16–20 weeks	24	0.7341 ± 0.28
20–24 weeks	18	1.3008 ± 0.23
24–28 weeks	16	2.136 ± 0.3
28–32 weeks	2	3.4471
New born[a]	1	4.5621
2 days	2	4.748
18 days[b]	1	3.7116
7 months	1	3.4211
3 years[c]	1	4.7264
4 and ½ years[d]	1	3.8876
Adult		8.0

Results are expressed in mean and standard deviation for the number of fetuses indicated in parenthesis

[a]Premature and 12 h of age

[b]Died of Paralytic ileus

[c]Vitamin deficiency Patient

[d]Died of Encephalitis

The weight of the organ in mg. is expressed per 100 g of body weight and results are plotted in a graphic form (Fig. 6.17). There is a steep decline in the early gestation period. The decline is not uniformly maintained throughout the gestation period.

The Growth in Mass of the Thymus

Detectable thymus was observed earliest in about 8 weeks of gestation when the fetus weighed about 1 g. In weeks earlier than 8, the thymus cannot be identified as an organ even under a magnifying glass. The thymic weight was expressed as mg/100 g of body weight. Table 6.10 shows some of these anthropometric measurements. Group A included 39 fetuses varying from a body weight of 1.3–14.7 g. In many of these fetuses, especially of in the smaller ones, thymic tissue could not be dissected. In all fetuses weighing more than 5 g,

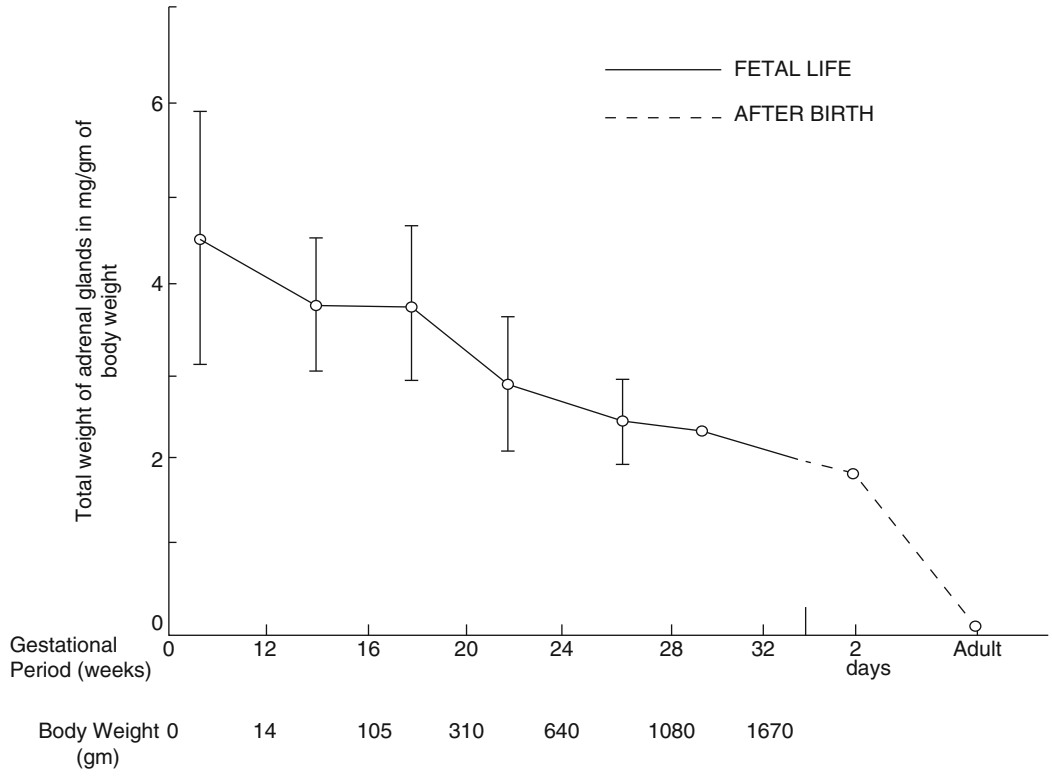

Fig. 6.13 Weight of adrenal glands mg per gm body weight/gestational age

Table 6.8 Growth of the testes in developing human fetus

Gestation period (weeks)	No. of sample	Bodyweight (gm)	Weight of testes (mg) mean ± S.D			Tissue weight in mg/100 g of body weight mean ± S.D
			Right	Left	Total	
9–12	11	5–11	4.5 ± 2.0	4.0 ± 2.0	8.5 ± 3.0	133.16 ± 36.05
3–16	24	22–80	12.0 ± 4.0	9.0 ± 4.0	21.0 ± 8.0	50.75 ± 17.35
17–20	31	130–250	39.0 ± 12.0	36.0 ± 14.0	75.0 ± 23.0	40.28 ± 2.89
21–24	24	350–450	70.0 ± 9.0	64.0 ± 11.0	134 ± 20	30.42 ± 2.60
25–28	13	700–1,000	93 ± 11	87 ± 9	180 ± 19	21.70 ± 1.42
29–32	4	1,100–1,400	132 ± 12	0.113 ± 20	245 ± 31	20.06 ± 0.46
33–36	1	1553.00	161	157	318	20.47

thymic tissue is dissectable. The organ formed 52 mg/100 g of body weight in this group. Group B included 28 fetuses of body weights varying from 15 to 100 g. The thymic weight expressed in mg per 100 g of body weight was 77 mg. This was higher than the thymic weight of fetuses of Group A. The relative growth of thymus was thus more in this period than the previous. Group C included 39 fetuses of body weights varying form 100–300 g. The relative rate of growth of the thymus in this period was still higher than that of fetuses of group B, in as

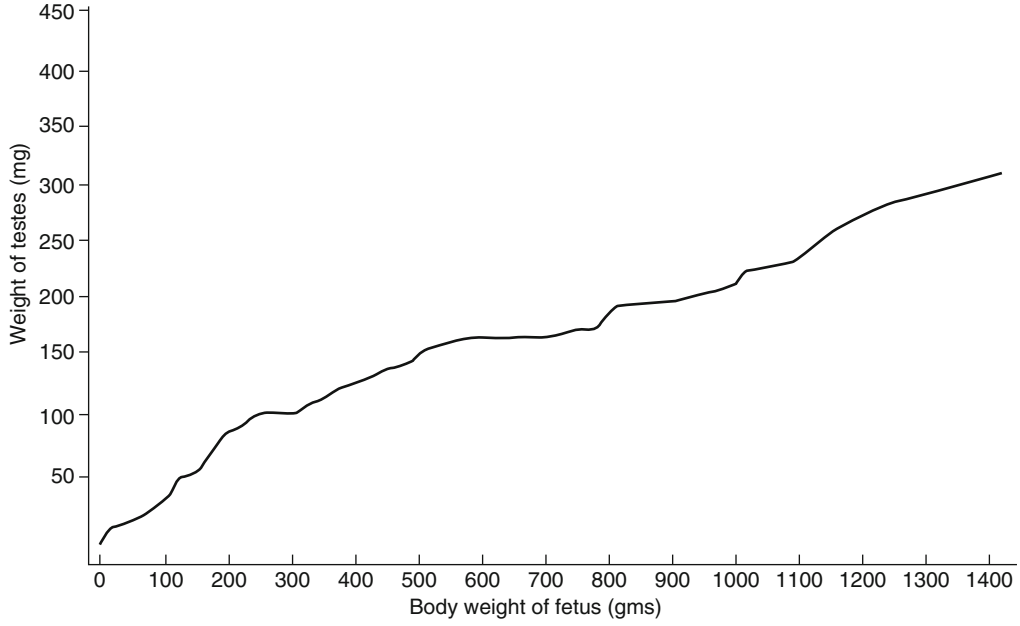

Fig. 6.14 Weight of testes plotted against weight of fetus

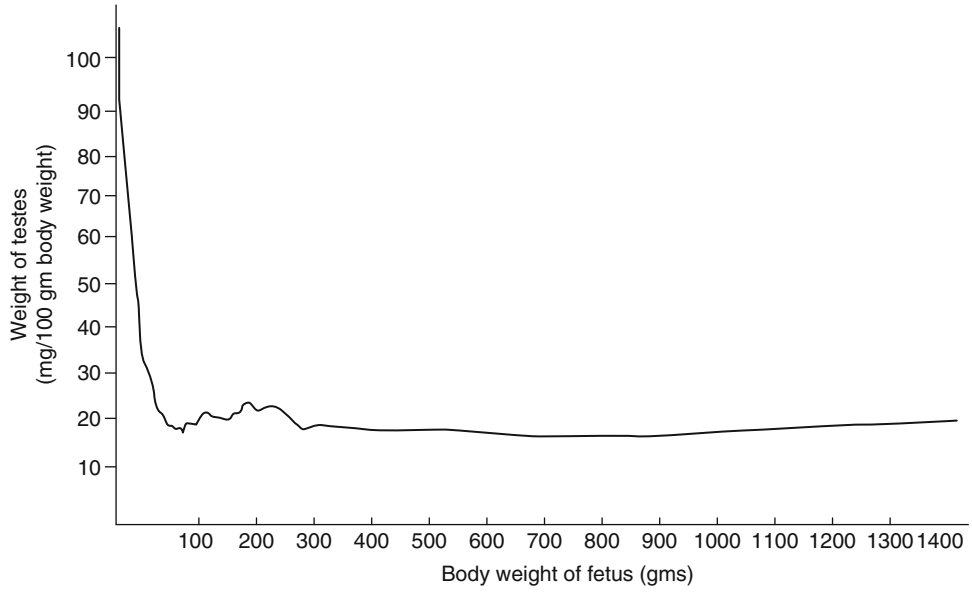

Fig. 6.15 Relationship of fetal testes/body weight

much as it formed 136 mg per 100 g of body weight in contrast to 77 in group B. This increase in the relative growth of the thymus in proportion to body weight was maintained throughout the period of gestation. We could study foetuses upto 28 weeks; it was highest in the

Table 6.9 Growth of the ovaries in developing human fetus

Group and gestation period (wks)	No. of sample tested	Body weight in gms	Weight of ovary in (mg) mean +/− S.D			Tissue weight in mg/100 g of body weight mean ± S.D
			Right	Left	Total	
9–12	13	5–11	3.0 ± 1.5	3.0 ± 1.5	6.0 ± 3.0	100.30 ± 43.76
13–16	38	22–80	15.5 ± 8.0	13.6 ± 6.0	29 ± 13	49.79 ± 8.61
17–20	38	130–250	38 ± 8.0	34 ± 7.0	72 ± 15	40.97 ± 6.83
21–24	26	350–450	81 ± 9.0	72 ± 9.0	154 ± 15	35.74 ± 4.39
25–28	10	700–1,000	95 ± 6.0	105 ± 35	0.200 ± 30	23.97 ± 2.77
29–32	4	1,100–1,400	110 ± 7.0	123 ± 12	234 ± 8	17.64 ± 1.48
33–36	1	1553.00	152	149	298	19.10

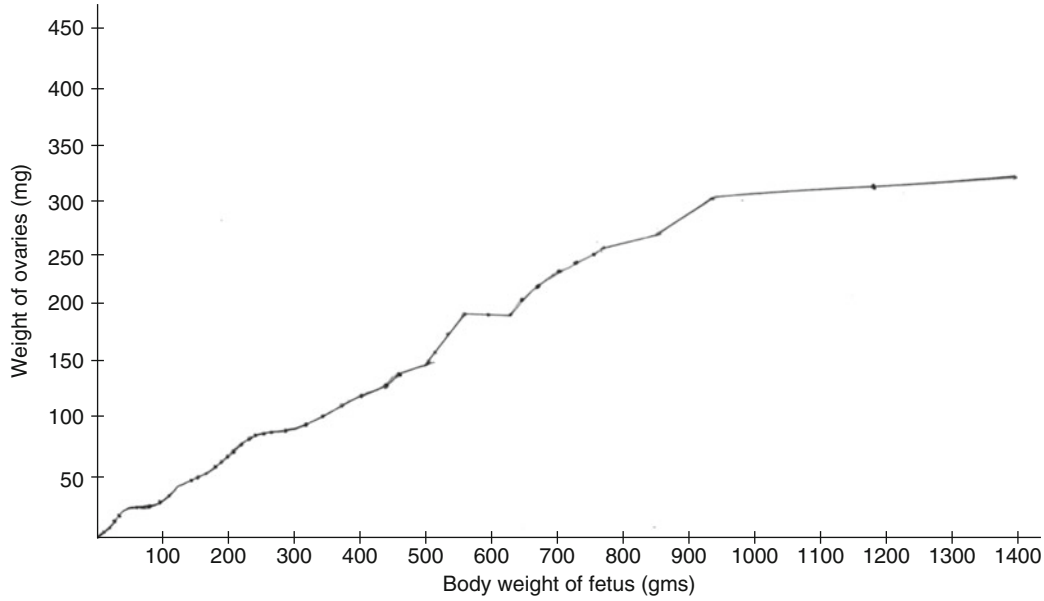

Fig. 6.16 Relationship of fetal ovary and fetal body weight

fetuses of large body weight. This is in contrast to many other organs like the brain and liver which constitute a more or less constant proportion of body weight except in the case of very early fetuses. Since the weight of the thymuses is expressed per 100 g of body weight, it can be inferred that with progress of gestation there is an absolute as well as a relative increase in the mass of the thymus. However one thing must be mentioned in this context. There were occasional fetuses in all the groups in whom the thymic weight was not as high as would be expected from the body weight. In almost all cases we could predict the weight of the liver or

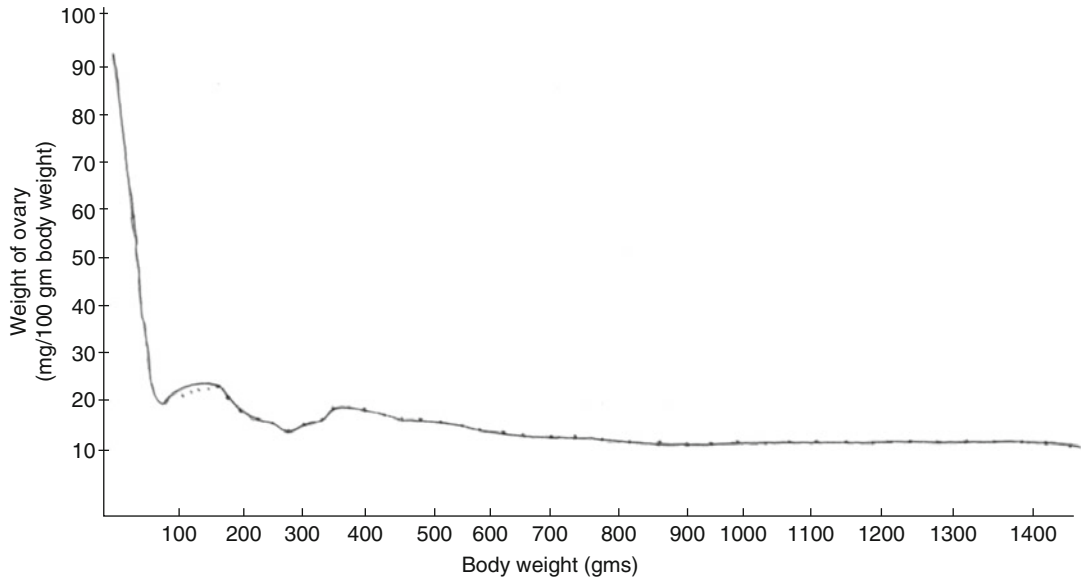

Fig. 6.17 Individual weights of combined ovaries in mg plotted against the weight of the fetuses in gms

Table 6.10 Growth of the thymus in developing human fetus

Gestational period (weeks)	Body wt. of fetuses (gms)	Thymic wt. in mg/100 g body weight
8–12 (39)	1.3–14.7	52.2 ± 8.39
12–16 (28)	15–100	76.6 ± 5.52
16–20 (39)	100–300	135.8 ± 7.66
20–24 (24)	300–580	218.6 ± 16.53
24–28 (19)	580–1,080	362.3 ± 24.23

The results are expressed as mean and S.E.M. of the number of fetuses indicated in parenthesis

the brain or the kidney from the body weight but we could not do so in the case of thymuses.

Figure 6.18 shows the relationship between the body weight of the fetus (in gm) and the thymic weight (in mg.). It is apparent that there is no straight line relationship between the body weight and the thymic weight. The rate of growth varies at different periods of gestation.

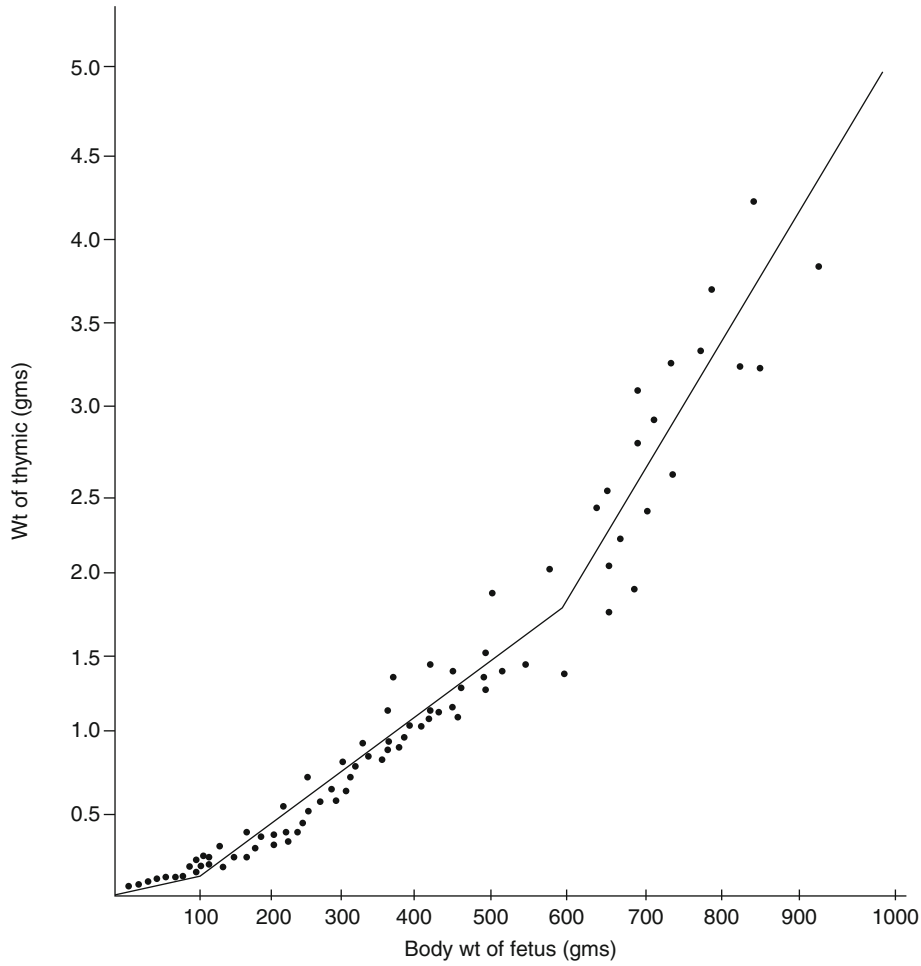

Fig. 6.18 Relationship between thymic weight and body weight of fetus

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Human Fetus: Carbohydrate Metabolism

7

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Introduction

Glucose is the unit of carbohydrate metabolism. All the three classes of carbohydrates mono, di and polysaccharides ultimately are converted to glucose and then metabolized. Some sugars join the common metabolic pathway of anaerobic glycosis. Except under special circumstances, for e.g., in galactosemic children there is no dearth of myelin on delivery. Therefore, the baby must have utilized galactose form the maternal circulation. In adult life, when the supply of glucose is not forthcoming like in the postprandial period, stored glycogen is broken down to glucose. Synthesis of glycogen is a function of the period when glucose is being absorbed form the gut. The state of glycogen synthesis and the breakdown becomes important in fetal life. In fetal life, however, the fetus is constantly supplied with glucose from maternal circulation. There is no need for glycogen to be synthesized and broken down in order to supply glucose to the circulation.

The history of glycogen synthesis and break down forms a very interesting part of the effort of many scientists.

In the inimitable word of Haldane, "Bernard was the first to formulate the extremely fruitful idea that the blood of a living animal in an internal medium is kept remarkably constant as regards its physicochemical condition by the co-ordinated influence upon it of the various organs of the body." The term internal medium, the milieu interieur, has been associated with the name of Claude Bernard [1], a pupil of the leading French physiologist Francois Magendie. Although Charles Robin [2] might have used the term earlier than Claude Bernard in his book, "*Treatise of Anatomical and Physiological Chemistry, Normal and Pathologic*," Bernard contemplated and discussed the idea of the internal environment for over 20 years and gradually added new facts of

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meaning to the idea of the milieu interieur. Almost at the same time P. Fluger [3], Frederick [4] and Riched [5] contributed immensely to the idea that internal environment is maintained by virtue of dynamic self-regulating mechanism, stability and constancy in the face of changing environment. Claude Bernard [1] said, "it is the fixity of the milieu interieur which is the condition of free and independent life; all the vital mechanisms, however varied they may be, have only one object, that of preserving constant the condition of life in the internal environment." According to him, the conditions which must be maintained constant in the fluid matrix of the body in order to favour freedom from external limitation are water, temperature and nutrient (including sugar). The concept of homeostasis was further elaborated by Canon [6]. The storage and release of carbohydrate as a means of keeping the blood sugar at a constant level under changing environmental conditions [7] was realized. Hansen [8] pointed out that there were normal oscillations in blood sugar occurring within a narrow range. Such insight and intuition in the 1920s were a real marvel when we realize that the methods of laboratory investigations were crude and rather imprecise in those days. That in the milieu interieur the blood sugar was maintained at a reasonably constant level was stressed by many authors ever since Otto Folin introduced the method of blood sugar estimation as a clinical laboratory technique. Thus it was found that after a meal rich in carbohydrate the bold sugar started to rise upto approximately 30–40 mg% above the fasting level at one and half hours and returned to the fasting level at two and half hours and remained at the level for a considerable period of time. During this period it was gradually realized that the human body was an organized system and an organized system required expenditure of energy in order to prevent the organization form spontaneous natural disorganization. It was found out that the energy was chemical in nature and was ultimately derived from the hydrolysis of nucleoside triphosphates. The synthesis of nucleoside triphosphates occurs mainly in the respiratory chain during operation of the tricarboxylic acie cycle. The tricarboxilic acid is formed by conjugation of oxaloacetic acid with acetyl coA. The Ac coA is derived from the decarboxlation of pyruvic acid. The pyruvic acid is produced by anaerobic glycolysis of glucose. There are some organs of the body which are obligatorily dependent on a supply of glucose. Glucose must, therefore, be supplied to these organs constantly. The supply line is through the blood. The blood must therefore carry a more or less constant amount of glucose at all times.

This concept of homeostatic regulation of internal environment in the face of constantly changing external environment was forcefully advanced by Claude Bernard in the later half of the nineteenth century, as mentioned earlier. Subsequently it was gradually realized that all the chemical reactions in our body occurred at constant body temperature and required presence of catalysts. These biocatalysts were found to be the different enzymes having definite specificities with regard to the substrates they catalyze. These enzymes regulate the level of metabolites found in an ever changing system, wherein the metabolites enter and leave the system in a finely and delicately controlled manner. Organic reactions are reversible. Since the enzyme catalyzed reactions are organic in nature, it is to be expected that they may occur in both directions depending upon the energy consideration in the system. Moreover, most of the bodily reactions are in a flux, i.e., sequential, the products of one reaction are substrates for other reactions and thus the flow may virtually be unidirectional under a particular set of conditions. Hence in order to maintain the level of a metabolite at a particular concentration it is required that constant rate of input into the system is maintained as also the same rate of output out of the system.

When we come to consider the maintenance of a more or less constant level of blood sugar, we are confronted with a number of changing external conditions which tries to offset the maintenance of a constant level.

Firstly, we should be aware of the fact that blood sugar is never constant. For example, after a meal containing carbohydrates the blood sugar concentration rise to a level between 30 and 40 mg% higher than the fasting level at about an hour after the meal and return to the fasting level between two and two and a half hours after the meal. So there are periods in which blood sugar concertration is higher than the fasting level. However, the organisms tend to adopt to the high blood sugar by

distributing it between the various fluids and metabolizing it either by catabolic reactions or by anabolic reactions. Conversely if food is withheld from our system, substances are broken down and mobilized in order to produce glucose and thus keep the blood sugar from falling. Therefore, by the statement "blood sugar concentration is kept constant", it is implied that blood sugar is kept within a certain level which varies depending upon various external conditions playing on the system.

In the human body the brain is dependent on the energy provided by glucose oxidation. Therefore the brain must always be supplied with glucose. In postnatal life, in the postprandial period glucose is added to the system by Glycogenolysis and Gluconeougenesis.

The human fetus is supplied with glucose from the maternal circulation. There is no necessity for Glycogenolysis and Gloconeogenesis.

Therefore it was considered important to study the glycogen content of the liver and muscle in different gestation period. This was part of the experiments done on the human fetus, mentioned in Chap. 6, by our group of researchers led by Prof. K.L. Mukherjee, at SSKM Hospital, Calcutta, from 1977 onwards.

Blood Sugar Concentration in Fetal and Maternal Blood

The mothers from whom the fetuses were obtained had opted for Medical Termination of Pregnancy which is legal under India's MTP Act, 1971, under specific circumstances. Due permission was taken from the hospital ethical committee and informed consent was taken from the mothers. Fetal blood was drawn from the heart within 10 min after the fetuses were taken out by hysterotomy. A sample of venous blood was obtained from the mother while she was under anesthesia, from the arm, opposite to the one, through which she was being infused with either saline or 5 % dextrose in saline. Blood sugar was estimated by the glucose oxidase method; in some cases the blood sugar was estimated by Folin and Wu's method in addition to the glucose oxidase method. Mothers were grouped in four groups of /4 week period beginning from 13 weeks of gestation. Table 7.1 show the results obtained by glucose oxidase method on the pairs tested in different groups. The mean fetal blood sugar was higher than the mean maternal level but the difference was not statistically significant. In approximately 80 % cases the fetal blood sugar was lower than the maternal but in 20 %, the fetal sugar level was higher than the maternal.

Table 7.1 Blood sugar concentration of fetal and maternal blood (glucose oxidase method)

Group	Gest period (wks)	No. of samples	Mean ± SD blood sugar in fetus (mg/dl)	Mean ± SD blood sugar in mother (mg/dl)
A	13–16	8	80.6 ± 10.72	59.2 ± 11.09
B	17–20	10	87.8 ± 16.4	77.9 ± 15.7
C	21–24	6	114 ± 26.5	106 ± 20.9
D	25–28	1	35.0	62.0

Table 7.2 Relationship between the high fetal blood sugar and the different glycemic condition of the mother

	Fetal blood sugar higher than maternal (% of total)	
	Higher	Lower
Normogycemic mother	16	84
Potentially diabetic mother	48	52
Frankly diabetic mother	35	65

Since in all published literature on the relationship between fetal and maternal blood sugar concentration, the fetal blood was shown to contain sugar at a lesser concentration than the maternal one [9, 10], we tried to investigate the cause of the high fetal sugar found to be present in some cases. The glycemic condition of the mother, whether normoglycemic, potentially diabetic or frankly diabetic had any relationship with the high fetal blood sugar is shown in Table 7.2. The potentially diabetic mother refers to those mothers whose hyperglycemia became normoglycemia after the termination of pregnancy. Of the normoglycemic mothers, 16 % showed high blood sugar level in the fetuses. In potentially diabetic mother about a half showed higher blood sugar level in the fetuses than the mothers. In frankly diabetic mother about a third showed higher blood sugar concentration in the fetuses.

Normoglycemia:	Fasting blood sugar 4.5–5.5 m Mol/l and post prandial blood sugar less than 6.5 m Mol/l
Potentially diabetic:	Blood sugar higher than 6.5 M Mol/l during pregnancy but below 5.5 m Mol/l after puerperium
Frankly diabetic:	The diabetic state persisting after puerperium

Glycogen Content in the Human Fetal Liver

One of the important parameters which maintains blood sugar level in the adult is liver glycogen. Glycogenolysis provides glucose-6-Phosphate (through glucose-1-P) which undergoes hydrolysis to glucose. The glycogen contents of the liver of those human fetuses were extracted within 15 min after removal from the uterus. The fetal liver glycogen was found to be reasonably stable for upto 24 h after the fetus was taken out. Portions of fetal livers were cut at 0.25, 1, 12 and 24 h. after their removal from the uterus and their glycogen contents were estimated (Table 7.3). Results agreed within reasonable limits (the later values were more than 80 % of the 0 h values). Three methods of extraction were tried: hot 33 % KOH, hot water and cold TCA. Hot 33 % KOH yielded the highest glycogen content. Therefore, all subsequent investigations were carried out on hot alkali extracted liver. Glycogen was precipitated with alcohol upto 60 %. Two washings with 60 % ethanol were enough to isolate the glycogen. Final color reaction was carried out with anthrone reagent. Results were multiplied by 1.1 to compensate for the hydrolysis to glucose equivalent. Results are shown in Table 7.4. The data were statistically analysed with respect to significant differences between the liver glycogen contents of fetuses at different stages of gestation. At 9–12 weeks the liver glycogen was very little; especially, most fetal livers of 9 weeks hardly contained any glycogen at all.

The liver glycogen contents gradually increased from a mean value of less than 0.1 g% at 9–12 weeks around 1.8 g% between 21 and 24 weeks. The pairs were matched with regard to the mean of similar glycogen contents in order to compare the values statistically. The increase of liver glycogen contents between the fetuses of different gestation groups was highly significant.

Table 7.5 shows that the glycogen content of skeletal muscles was again lowest at 9–12 week (0.4 %). But from 13 to 24 weeks the muscle glycogen content remained more or less steady at around 1 %. As a result, the difference in skeletal muscle glycogen content between fetuses at different groups was only significant between the earliest gestation groups and between no other groups. If we compare the glycogen contents of the liver and muscles in the corresponding gestation periods, we find in Table 7.6 that skeletal muscles had higher glycogen contents than the liver at

Table 7.3 Glycogen stability in human fetal liver (gm/100 g)

Time	Temperature of storage	
Hours	20 °C	–5 °C
0.25	1.66	1.89
1	1.59	1.77
2	1.90	1.59
12	1.30	1.87
24	1.55	1.62

Table 7.4 Glycogen content human fetal liver at different gestation period

Group	Weeks	Number of samples	Mean gm%	S.D.	P value	Remarks
A	9–12	14	0.08	0.09	<0.001	Highly significant
B	13–16	32	0.57	0.54	<0.001	Highly significant
C	17–20	32	0.93	0.36	<0.001	Highly significant
D	21–24	19	1.83	0.70	0.001> P <0.005	Highly significant

Table 7.5 Glycogen content of human fetal muscle at different gestation period

Group	Weeks	Number of samples	Mean gm%	S.D.	P value	Remarks
A	9–12	10	0.40	0.52		
B	13–16	10	1.07	0.29	<0.001	Highly significant
C	17–20	10	0.96	0.36	<0.500	N.S.
D	21–24	10	1.12	0.34	P >0.4 <0.2	N.S.

Table 7.6 Inorganic phosphorus content of human fetal liver at different gestation period

Age in week	Number of sample	Body weight (gms)	Liver weight (gms)	Total Pi (mg/100 g)
9–12	2	1–14	57.41 (mean)	0.47 (mean)
13–16	15	15–110	65.83 ± 4.82	3.12 ± 0.62
17–20	14	110–300	66.04 ± 4.89	7.18 ± 0.63
21–24	9	300–600	114 ± 6.7	16.00 ± 1.02
25–28	2	600–1,000	199.2(mean)	–
37–40	2	1,533	1,133.2(mean)	–
Adult	2	–	53.7	–

9–12 week. Thereafter the values were similar and later on the liver glycogen contents were higher than the muscles.

Inorganic Phosphorus Content in the Human Fetal Liver

Phosphorus is a very important constituent of the organ both as regards its own metabolism as well in relation to the handling of glucose. Unlike the adult whose dietary intake contains abundance of phosphorus, the intrauterine fetus depends entirely on the maternal supply of inorganic phosphorus through its placental circulation. It was considered, therefore of interest to study the magnitude of phosphorus content in fetal organs at different periods of gestation. The human fetal liver was studied, and the results are shown in Table 7.6. The results are expressed as mg of inorganic phosphorus per 100 g of liver weight. From 9 to 21 weeks the total inorganic phosphorus contents were higher. Total liver inorganic phosphorus content were plotted against the body weight of the fetuses. There was an almost straight line relationship between the two parameters upto a body weight of 300 g; thereafter the slope was steeper. The inorganic phosphorus content of the mitochondrial fractions was usually lower than that of the cytosolic fraction. Two adult livers could be obtained during the period of study. The inorganic phosphorus content was lower than that of the fetal liver.

Phosphorylase Activity in the Human Fetal Liver

Glycogen is broken down to glucose-1-p by the activity of the enzyme phosphorylase. Since Keq of the enzyme is close to 1, the activity of the enzyme is usually measured by the synthetic reaction, i.e., incorporation of glucose into the growing glycogen chain and consequent liberation of inorganic phosphate form glucose-1-p in 8,000 × g supernatant. Phosphorylase activity of the liver of a few fetuses was measured according to the method of Stalmans and Hers [11]. Results are shown in Table 7.7; the activity at 9–12 weeks was about a tenth of the adult liver activity of the adult livers. At 13–16 and 17–20 weeks the activity is about a fifth of the adult liver activity. According to Hers [12] the phosphorylase activity of adult human liver as obtained from liver biopsy, is 27.8 μ Mole pi/g tissue/min (mean value). Phophorylase activity of the skeletal muscle was estimated form crude homogenate of the thigh muscle (quadriceps Femoris). Results are shown in Table 7.8.

The enzyme activiry of the fetal muscle was much lower than that of adult muscle reported by Hers [13]. In fact adult muscle phosphorylase activity was about four times higher than that of the adult liver. In comparison, however the

Table 7.7 Phosphorylase activity of the human fetal liver at different period of gestation

Gestation period (wks)	Number of specimen	Enzyme activity μ Mole pi/g/tissue/min	Specimen activity μ Mole pi/g/protein/min
9–12	2	2.52	13.39
13–16	7	5.60 ± 0.46	21.94 ± 4.37
17–20	6	5.58 ± 0.68	16.21 ± 3.6
21–24	1	4.09	12.4
Adult[a]	4	27.8	–

[a]According to Hers [12]

Table 7.8 Phosphorylase activity of human fetal muscle at different period of gestation

Period of gestation (wks)	Body weight (gm)	Phosphorylase activity μ Mole pi/g T/min
10	4.3	1.70
14	35.2	0.58
18	187.0	1.75
24	549.0	4.6
Adult[a]	–	100

[a]According to Hers [12]

Table 7.9 Non specific activity of glucose -6 – pase in the human fetal liver

Gestation period (wks)	Mg pi/g/tissue/15 min (according to the method of Swanson)	Mg pi/gm/tissue/15 min (after prior incubation at Ph 5)
8	0.24	0
20	5.76	1.44

phosphorylase activity of the fetal muscle was lower than that of the fetal liver. The reported value of adult muscle phosphorylase by Hers [13] is 100 μ Mole Pi/g tissue/min. In other words the fetal muscle had very little phosphorylase activity.

Glucose: 6 Phosphatase Activity of the Human Fetal Liver

The hydrolysis of liver glucose -6-phosphate was carried out according to the method of Swanson [14]. The liver homogenate was carried out in maleate buffer (0.1 M, PH 6.5) at a concentration of around 50 mg per ml of liver homogenate. It was centrifuged at 600 × g and the supernatant was used as the enzyme source. It was soon realized that the activity of the enzyme which was observed in human fetal liver of earlier gestation period might be an artifact, possibly due to non specific phosphatases present in the homogenate supernatant. Accordingly and as suggested by Hers [13], the nonspecific phosphatase was inhibited by prior incubation of the enzyme at pH 5.0 in an acetate buffer and then the enzyme activity was determined. An example may perhaps, be cited in one of the cases where enzyme activity was found to be present by the original method of Swanson but it was shown to be absent when the nonspecific phosphatase activity was subtracted. Table 7.9 shows the result. In an 8 week old human fetus some measurable activity was present when it was estimated according to the original method of Swanson. But prior incubation at pH 5.0 inhibited the not specific phosphatase and no G-6-Pase activity was found in the liver at this period of gestation.

The method of glucose-6-phosphatase activity is based on liberation of inorganic phosphorus from glucose-6-phosphate, according to the method of Swanson [14]. Results are shown in Table 7.10. In the fetus of 9–12 weeks no glucose 6-pase activity was detected. Both activities, per gm tissue and specific activities of the enzyme increased progressively.

Earliest evidence of glucose-6-pase activity was demonstrated at 14.5 weeks of gestation. From then on, the activity increased progressively, but even at 24 weeks of gestation the

Table 7.10 Glucose – 6 – pase activity of activity of human fetal liver at different period of gestation

Gestation period (wks)	No. of samples	Activity µMole pi/g T/min	Sp. activity µMole pi/g protein/min
9–12	14	Nil	Nil
13–16	8	0.248 ± 0.017	2.53 ± 0.14
17–20	6	0.764 ± 0.49	5.22 ± 0.34
21–24	6	1.269 ± 0.08	6.488 ± 0.359
Adult	4	2.5	9.6

enzyme activity was lower than that found in adult livers.

Since glucose-6-pase activity was not found in livers of human fetuses in the earlier weeks of gestation, it was considered advisable to study if the placenta could serve the purpose of the liver in this respect. Accordingly the whole placenta was homogenized in a waring blender in the cold and aliquots of placental homogenate were centrifuged at 500 × g; the supernatant was used as the enzyme source. Glucose-6-pase was estimated by the method of Swanson and about 5 mg tissue equivalent was used in the assay. Results are shown in Table 7.11. In the placenta of earlier gestation period, the specific activity of glucose-6-pase was much higher than that of later gestation period.

Some properties of the fetal liver glucose-6-pase activity was studied in the microsomal pellet (105,000 × g pellet). The pellets were resuspended in cacodylate buffer (0.15 M, Ph 6.5) and enzyme activity was determined by the method of Segal and Washko [15]. Result are shown in Table 7.12. The specific activity of the microsomal pellet was about twice that of the 5,000 × g supernatant. The 105,000 × g supernatant had no activity.

The 105,000 × g pellet was used to measure the activity of the enzyme by the above method with and without the presence of certain compounds and results are shown in Tables 7.13. The end product measured was glucose by the method of Nelson and Somogyi [16]. The full system showed an activity of 6.1 µ Mol glucose liverated/ mg protein per min. Inorganic phosphate at 100 Mol concentration produced a complete inhibition. Similarly ATP at 0.2 Mol concentration produced a complete inhibition.

Table 7.11 Glucose – 6 – pase activity of the human placenta at different period of gestation

Gestation (wks)	Fetal body Wt. (g)	Placental g-6-pase activity µMole pi/g protein/min
14.5	48.2	67.8
19	219	2.14
19	237	4.98

Table 7.12 Glucose – 6 – pase activity of the human fetal liver in different subcellar fraction

Gestation period (wks)	Fraction	Glucose – 6 – pase activity µMole pi/g protein/min
21	5,000 × g sup	4.44
–	105,000 × sup	Nil
–	105,000 × pellet	7.87

Table 7.13 Effect of inorganic phosphate and ATP on the activity of glucose – 6 – pase of human fetal liver

Addition of	Concentration (µ M)	Sp. activity of G – 6 – pase µ M g/mg pro/min
Inorganic phosphate	25	6.1
–	50	2.0
–	100	0
ATP	0.20	0
–	0.50	0
–	1.00	0

Fructose 1,6: Diphosphatase in Human Fetal Liver

Fructose 1,6 diphosphatase is one of the key enzymes of gluconeogenesis and highest activity is present in gluconeogenetic tissues, e.g., liver [17]. The activity of the enzyme is subjected to modifiers and regulators [18].

Since one of the objectives is consideration of the factors producing gluconeogienesis, the activity of the enzyme was measured in the livers of human fetuses of different gestation periods. The method used was a modification of Lahiri et al. [19]. The activity was measured at pH 7.5 and pH 9.5; the results are shown in Table 7.14. The activity as measured at pH 7.5 was always less than the activity at pH 9.5 at all stages of gestation. The developing

Table 7.14 F – D pase activity of human fetal liver at different periods of gestation of 10 K fraction

Gestation period (wks)	Number of sample	F – D pase activity at pH 7.5 and pH 9.5 μ Mole pi/g pro h	
13–16	4	0.20	0.38
Do	–	0.37	0.86
Do	–	0.57	0.60
Do	–	0.17	0.25
17–20	2	0.49	0.60
Do	–	0.33	0.43
21–24	2	0.17	0.20
Do	–	0.19	0.49

human fetus receives glucose continuously through the placental circulation *vide* Biswas et al. [20]. However, apart from the supply of glucose from the mother, demonstration of FD Phase activity in liver within 8–32 weeks of gestation evokes gluconeogenic possibilities, [21]. Participation of the gluconeogenic enzyme during development for either fetal or neonatal energy homeostasis has been documented in several mammals [22]. Nevertheless, a clear insight regarding the fetal gluconeogenic capacity in mammals, particularly in human, is yet to be elaborated.

Human fetal liver fructose 1,6 – Diphosphatase has been purified about 200-fold to homogeneity at pH 7.5. The purification procedure for the enzyme was applicable throughout human fetal gestation. The purified enzymes recorded an approximate Km of 1.603×10^{-5}M for FDP and 1,6-biphospahte at pH 7.5. The enzyme could hydrolyze sedoheptulose – 1,7 – biphosphatase also. The molecular weight of the native enzyme as determined by gel filtration was approximately 146,000. The enzyme was Mg^{2+} depended, which could be replaced by Mn^{2+}. Ca^{2+} was absolutely inhibitory. The Monovalent cations had no effect except Li^{1+} which inhibited the enzyme activity at pH 7.5. Histidine, citrate and EDTA were found to be stimulatory at pH 7.5, with optimal stimulation at 1 mM, 2 mM and 0.04 mM respectively. Zn^{2+} exhibited both stimulation and inhibition of the human fetal liver FDPhase. However, optimum stimulatory concentration of histidine partially recovered the enzyme activity at pH 8.5 from Zn^{2+} inhibition. 5^{-} – AMP inhibited the enzyme activity at pH 7.5 and inhibition was temperature dependent. The inhibition of the neutral FDPhase activity by 5^{-} – AMP was potentiated by fructose -2-6 – biphosphate. The fructose -2-6 – biphosphate inhibition of the enzyme in absence of 5′AMP was found both at pH 7.5 and pH 8.5 but not at pH 9.5.

Discussion

A number of studies have been carried out on blood sugar concentration of fetal and maternal blood in different species [9, 10, 23–25] and in man. Most of these studies has reported lower sugar concentration in fetal blood than in the maternal. This is logical since transport of sugar across the placental circulation has been thought to be facilitated by diffusion type involving a carrier [26, 27]: and the process does not involve expense of energy. The carrier mediated transport is through GAP junction. Subsequent to the binding of the ligand to the receptor the pore is widened to transport the ligand. Since it does not involve waste of energy, the transport must be down a concentration gradient. Therefore, in order that glucose be transported to fetal circulation the concentration in the maternal circulation must be higher than in the fetal. It is rational, therefore that most studies found this to be so. The greater the difference in the maternofetal concentration the higher the transport. There were few studies on the blood sugar concentration in the human uterine artery and umbilical vein simultaneously because of obvious ethelical consideration. Furthermore it is difficult to measure the uterine and umbilical blood flows in order to calculate the rate of transport of sugar across the placental barrier. Such studies have been carried out in lambs [28]. By means of antipyrine steadystate diffusion technique, simultaneous umbilical and uterine blood flows were measured. Fetal oxygen consumption was found to be relatively constant among species whereas the metabolic rates were found to depend on the body size [29]. Glucose/oxygen quotients across the umbilical circulation during the last third gestation period in lambs was found to be 0.5 which was interpretated to mean that only 50 % of

oxygen consumption in fetal lamb could be accounted for by umbilical glucose uptake.

Blood sugar concentration in the human fetus is different in different parts of the fetal circulation [30]. Thus, in the umbilical artery the fetal sugar concentration is almost always lower than the maternal sugar. However, in blood drawn from the heart the concentration is occasionally higher; this may be due to a number of reasons. Blood flowing through the liver may acquire some sugar by glycogenolysis or gluconeogenesis. Furthermore, some amount of delay was inevitable between hysterotomy and drawing of fetal blood. During this interval, the fetus is totally anoxemic, although the heart was still found to pump blood when the fetal thorax was opened. What effect the anoxemia has on glucose homeostasis it is difficult to say. On the one hand, tissues will try to derive energy by increased glycolysis. On the other hand, the lactic acid may be utilized to produce glucose by gluconeogenesis. The ultimate effect on blood sugar concentration may be provided by the relative rate of glycogenolysis and gluconeogenesis.

The percentage of fetal blood sugar higher than the mother, was more in diabetic state either overt or gestational. In maternal hyperglycemic condition, excess glucose in transferred to the fetus which develops hyperinsulinemia [31]. Elevated insulin level in the fetal plasma may lead to increased glycogen synthetase activity [32]. However the response is slow and not immediate. Some fetuses may not have developed the insulin receptor fully and fetuses may vary in their time sequence of development of the insulin receptors. Maternal hyperglycemia is more amenable to be regulated by insulin response than fetal. Thus in potentially diabetic mothers, more instances of higher fetal glycemia than maternal may be found. Glucose homeostasis in fetuses of diabetic mothers is therefore, more unstable than in nondiabetic mothers. Diabetes is associated with increased production of ketone bodies.

If the ketone bodies pass to the fetal circulation they may be preferentially utilized by the fetus so that the glucose remains underutilized. The underutilization of glucose especially if associated with some gluconeogenesis may explain the higher glucose concentration in some fetuses than in their mothers.

Most of these studies are one point studies, i.e., blood sugar concentrations have been determined once only, maternal sugar, either when she was on an empty stomach or during the operation of hysterotomy, and the fetal, during the dissection. Such studies leave much to be desired. Although we can imagine organismal homeostasis to be operative, the homeostasis would take place over a time period and not instantaneously especially in the mother during stress or in the developing fetuses. In the meanwhile a number of environmental alterations are occurring which may have an effect on glucose homeostasis. One of these alterations is the operation of hysterotomy and attendant infusions of saline or other fluids during the operation. Ordinarily, an infusion of either saline or dextrose at around 2 ml per min does not produce an alteration of blood sugar concentration in a normal adult person. But the conditions may not be called normal in pregnant mothers undergoing the operation of hysterotomy. The relationship between fetal and maternal blood sugar in mothers being infused with either saline or 5 % dextrose was more or less the same. We may, perhaps, imagine that maternal homeostasis under these circumstances led to no relative alteration in the maternal sugar concentration to have corresponding effect on the sugar concentration in the fetus.

Serum inorganic phosphate concentration of mothers on an empty stomach and during the operation of hysterotomy were found to be more or less the same, although the blood sugar concentrations during the operation was higher than the concentrations on an empty stomach. A rise of blood sugar after a glucose tolerance test is attended with fall of serum inorganic phosphorus. It is attributed to the entry of sugar into the cells, consequent to the stimulation of insulin release. The sugar would form the different phosphorylated compounds thus utilizing the serum inorganic phosphorus. The rise of blood sugar during the operation of hysterotomy is perhaps produced by glycogenolysis. The utilization of phosphorus during the phosphorolysis is perhaps

compensated by the glucose-6-phosphatase activity. The net result under these circumstances would be no change in the serum inorganic phosphorus. Furthermore, the increased blood sugar level during the operation of hysterotomy may not be attended with increased utilization of sugar in the cells because of the stress of anesthesia and the operation. The serum inorganic phosphorus would, therefore, remain unchanged in the mother. Fetal serum inorganic phosphorus was almost always higher than the maternal. The transport of inorganic phosphorous should be receptor mediated or coupled to some other metabolic transport, from the maternal to the fetal side: as otherwise a reverse transport should occur since the fetal serum inorganic phosphorus is at a higher concentration than the maternal. Utilization of inorganic phosphorus is mainly in the formation of the various organic phosphates including the high energy phosphates and in the deposition of inorganic phosphates in the skeletal tissues. We can assume both these processes to be very active in the growing fetus. So, other process remaining unaltered, the serum inorganic phosphorus in the fetus should be lower than the maternal. An adult organism gets rid of the excess inorganic phosphorus through renal excretion. Fetus is a closed system within the mother. It cannot excrete inorganic phosphorus into the outside environment. It only can utilize the placental circulation to get rid of the excess inorganic phosphorus. Perhaps, this process is not very efficient and there is an accumulation of serum inorganic phosphorus in the fetus. The serum inorganic phosphorus is also high in the newborn and young children. In them also, renal excretion may not have matured enough to excrete excess inorganic phosphorus. Liver glycogen content of human fetuses were studied by Szendi [33], Villee [23, 34], Gennser et al. [35] and Capkova and Jirasek [36]. Our results vary somewhat from the previously published results. Capkova and Jirasek found about 0.34 % of glycogen at 9 weeks gestation, whereas we found a much lower amount (less than 0.1 %) at this period. The reason is unclear the mother, in our case, came from a lower socioeconomic status but did not differ very much in nutritional status from other mothers in respect to body weight, height and skin fold thickness. Furthermore, the studies of Capkova and Jirasek stressed on two spurts in respect of glycogen contents at around 14 and 17 weeks. We however, found liver glycogen content to rise throughout the period of gestation upto 22 weeks. Our results are in agreement with those of Villee [23] who also found a proportionate increase in liver glycogen content from 9 to 22 weeks gestation. In contrast to the liver, the muscles maintained a more or less steady concentration of glycogen, except during the early gestation period. Capkova and Jirasek [36] differentiated three types of glycogen in human fetal organs. In one type, glycogen content is high at first and decreases with progress of gestation. In the second type, glycogen content increases with gestation and in the third type, the glycogen content does not show any change during the course of gestation. Heart muscle and kidneys belonged to the third type. We found that skeletal muscle glycogen belongs to the third type. The fetal liver glycogen belongs to the second type, i.e., it increases with increase of gestation. Under steady concentration of glucose as it is obtained in the human fetus, why should the liver and muscle behave in a different way with respect to glycogen content? The liver is an active metabolic organ even in the fetus, i.e., it systhesizes compounds which are necessary for fetal homeostasis; the fetal muscle, on the other hand, is more concerned with its own development rather than in the development of any other organ. The glycogen content is perhaps a reflection of the different fundamental roles played by the two organs in fetal life.

Normal glycogen possessing the usual branched structure in the presence of iodine has maximum absorption at higher wave lengths (460 mu) which shifts to lower values in limit dextrin (390 mu). Furthermore, amylopectin in the presence of iodine has maximum absorption at around 520 mu. The thrice precipated human fetal liver glycogen in the presence of iodine had higher absorption at 460 mu than at 520 mu; this shows that the liver glycogen had a branched structure and is not like that of amylopectin. Thus the glycogen synthesized in the fetal liver must have the branching enzyme creating a large num-

ber of nonreducing ends which are the sites of action of both glycogen phosphorylase and systhetase. In this way both synthesis and breakdown of glycogen is enhanced.

Fetal liver glycogen was found to be unusually stable. Whereas adult liver glycogen was degraded rapidly at room temperature, fetal liver glycogen was degraded less than 8 % only at room temperature for 24 h. Perhaps lysosomal activation in fetal liver was not profound as in adult liver. Lysomal enzymes were found to be present in fetal liver but the enzyme activity was much lower than in adult liver. The glycogen content of the fetal liver can, therefore, be considered representative of actual in vivo glycogen content.

There was no correlation between fetal blood sugar and fetal liver glycogen content. One of the ways in which glucose homeostasis is obtained in the adult is by glycogenesis. Thus the rise of blood sugar following a meal stimulates insulin release from the pancreas leading to increased glucose penetration in the muscle and stimulation of glycogen synthesis both in the muscles and the liver. In the fetus, however the glucose concentration is most probably uniformly held constant: in other words there is no fluctuation in the concentration. So presumably there is no equivalent post absorptive hyperglycemia and no stimulation of insulin release. Thus glycogen synthesis in the fetus is not regulated by sugar concentration. Rapidly dividing cells, e.g., in fetal life, may be associated with low cyclic AMP, which in turn may favour the co- valent modification of glycogen synthetase, which synthesizes glycogen at a rate independent of blood sugar concentration and at its own characteristic rate.

In adult the serum Pi is regulated by the excretion of phosphorus in the urine. The absorption is regulated by 1:25 dihydroxy cholecalciferol and the excretion by parathyroid hormone. In the human fetus the excretion of phosphorus through the renal tubules cannot be regulated. The supply of phosphorus is not discontinous as in adults but occurs uniformly through the placental circulation. Under these modified circumstances the serum Pi content of the human fetus from 9 to 20 weeks of gestation is about three times that of the adult. One of the reasons must be that the human fetus passes very little urine and therefore, cannot excrete much phosphorus. The only avenue left is backflow through the placental circulation. The backflow is probably prevented by the gap junctions possessing vectorial transport, i.e., from mother to fetus only and not in the reverse direction. The serum Pi is related to sugar metabolism in various ways. Thus the element is necessary for formation of the various hexose phosphates and other intermediates inside the cell, once glucose enters it. If there is significant glycogenolysis, Pi is utilized to produce glucose-1-p. There are various other uses of Pi. One of the main uses is deposition of calcium phosphate in the developing and growing skeletal system as hydroxyl apatite, the chief salt being tricalcium phosphate. The maintenance of high fetal serum Pi indicates vectorial properties of Pi transport across the placenta. The relative independence of the level of serum Pi on the face of the widely different blood sugar values in the fetus indicates that the two levels are independently regulated.

Formation of glucose from glycogen or lactic acid or glucogenic amino acids involves the ultimate enzyme G-6-Pase.In connection with gluconeogenesis in human fetuses, therefore, the enzyme has received considerable attention. Incubation of human fetal liver from therapeutic abortion with labeled substrates showed that the liver can produce glucose from pyruvate and glycerol in vitro [34]. The low G-6-Pase found in our series may be commensurate with high G-6-P dehydrogenase in the hexose monophosphate shunt pathway. Thus more of the pentose phosphates may be produced and the ribonucleotide diphosphate reduced to the deoxyribonucleotide diphosphate by the NADPH generated in shunt pathway. Thus the tissues with high mitotic rates may be assured of a supply of the required intermediates by the operation started by the low G-6-Pase pathway. Furthermore, the presence of the G-6-Pase in vitro may not necessarily be synonymous with operation of gluconeogenesis as the translocase system for the G-6-Pase may be deficient in the human fetus at least in the early period of gestation. The enzyme was inactivated by both ATP and Pi in accordance with the usual properties of the enzyme.

Studies on the activity of the human fetal liver fructose 1:6 phosphates have been very limited. The gluconeogenic enzyme, of which this enzyme forms one of the key enzymes, has been studied in human, sheep, guineapig and baboon fetuses [24, 37]. A detailed study of these enzymes in the human fetus obviously could not be undertaken: most of such studies were carried out on subhuman primates [22, 38] and these studies furthermore, were confined to estimation of the enzyme activity in cell extracts from liver or kidney.

Glycogen content of the liver and muscles are broken down to glucose-1-phosphate by the enzyme glycogen phosphorylase. Fetuses of different species have different behaviours with regard to synthesis and breakdown of glycogen. They also behave differently with regard to phosphorylase activity. Thus in rats the activity of the enzyme begins to rise abruptly from about 3 days prior to birth; the rate of increase continues for another couple of days postpartum and then begins its decline to reach the adult activity [39]. The equilibrium constant of phosphorylase activity is close to 1. Under normal conditions prevailing in vivo, the product of glycogen and inorganic phosphate concentration far exceeds that of glucose-1-p, so that in vivo the enzyme exhibits Glycogenolysis only. The inorganic phosphate concentration in human fetuses is almost three times that of the adult. Hence, the enzyme activity is far in the direction of breakdown than synthesis.

The enzyme activity in human fetal liver from 13 to 20 weeks of gestation was more or less unchanged. We did not do estimation of active and inactive phosphorylase activities of fetal liver as was done in the case of rat [40]. We cannot say how much of the phosphorylase activities we measured were due to inactive or active enzymes, although it is possible that during manipulation after the fetuses were taken out, much of the otherwise inactive enzymes would have been converted to the active one [11].

Considering the very small amount of phosphorylase activities present in the human fetal liver upto 20 weeks of gestation, it may be concluded that the derivative activity is very limited in human fetal liver upto this age.

Summary and Conclusion

1. The relationship between human fetal and maternal blood sugar concentrations was studied in 25 normal fetuses of different gestation period and their corresponding mothers. In 80 % of these pairs the sugar content from fetal heart blood was lower that the maternal but, in 20 % it was higher than the maternal.
2. In the case of those fetuses whose blood sugar was higher than the maternal about half the mothers were pre-diabetic.
3. There was no significant relationship between the infusions the mothers were receiving at the time of operation and the blood sugar content of the fetuses.
4. The serum inorganic phosphorus contents of the fetal blood were higher (more than double) than that of the mothers. But these had no relationship with the relative glycemic condition of the fetomaternal pairs.
5. The glycogen contents of the fetal livers were analyzed. At 9–12 weeks the glycogen contents were very low; thereafter the values increased gradually form less than 0.1 g% at 9–12 weeks to around 1.8 g% at 21–24 weeks.
6. The fetal muscle glycogen contents were very low at 9–12 weeks and thereafter stayed at around 1 %.
7. The fetal liver glycogen had normal structure as judged by the iodine color and was unusually stable even at room temperature upto 24 h.
8. There was no relationship between fetal liver glycogen content and relative glycemic condition of the fetuses in the fetomaternal pairs.
9. The inorganic phosphorus contents of the liver were between 57 and 66 mg per 100 g tissue at 9–20 weeks and 114 mg per cent at 21–24 weeks.
10. Glucose-6-pase activity of human fetal liver was undetectable below 12 weeks of gestation. It was demonstrable thereafter and increased progressively but even at 24 weeks the activity was frequently less than that of the adult activity. Some experiments were undertaken to standardize the measurement

of activity of human fetal livers and the buffer systems, substrate concentration, homogenate aliquot inactivation at acid PH etc., were standardized.

11. The human placenta was found to possess Glucose 6-Pase activity at a higher level in earlier gestation period than in later.
12. The activity of fructose 1:6 biphosphatase in human fetal liver of different gestation periods was studied. The activity at pH 7.5 was found to be less than activity at pH 9.5.Measurable activity of the enzyme was detected from 11 weeks of gestation onwards. Some properties of the human fetal liver enzyme was studied like cytosolic location, heat treatment, and inhibition and activation by various organic compounds. Some attempts at purification of the enzyme were also undertaken.
13. The phosphorylase activity of human fetal livers of different gestation periods was studied. The enzyme activity throughout the gestation period was found to be very low.
14. The phosphorylase activity of human fetal muscles of different gestation periods was determined and compared to that of adult human muscles. The activity of the fetal muscles was found to be much lower than that of the adult muscles. In adult muscles the phosphorylase activity was higher than that of the liver but, in fetal muscles the reverse was found to be true.
15. These investigations tended to show that components are available in human fetal organs for gluconeogenesis but, it is doubtful if the process plays any significant role in the fetal system in vivo.

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8 Mucopolysaccharides, Water and Electrolytes of Human Fetal Organs

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Introduction

Structure of Mucopolysaccharide

A group of long chin heteroglycans or heteropolysaccharides consisting of N-acetylhexosamine, hexuronic acid or hexose as repeating units, occurring in animal tissues, is termed "Mucopolysaccharide" (MPS). Some of these polysaccharides are also polyelectrolytes, behaving as polyanionic substances due to the presence of free carboxylic (--COO^-) and sulphate (--$SO_3^=$) groups. Such acidic mucopolysaccharides (AMPS) mainly occur in connective tissues and universally contain acetylated hexosamine. Jeanloz [1] termed these MPS as Glycosaminoglycans (GAG) (Fig. 8.1).

The elucidation of the basic structure and some characteristic properties of at least seven different nitrogen containing heteropolysaccharide-protein complexes in the ground substance of connective tissue was established (Fig. 8.2).

Mucoproteins are in Stacey's terminology, carbohydrate protein complexes with a relatively high protein or peptide content. Ovomucoid, serum mucoprotein, pituitary hormones, and submaxillary gland mucin belong to this group.

The high proportion of carbohydrate (80 % of the total wt. in blood group substance) had led some authors [2, 3] to classify these substances together with hyaluronateprotein complex and Chondroitin Sulphate (CS)-protein complex, as MPS. The macromolecular structure of this protein carbohydrate complex has been studied by various groups of workers, e.g., Tsiganos and Muir [4] and Robinson [5] etc., and termed as proteoglycan.

Buddecke et al. [6] and Rosenberg et al. [7] and many other workers observed that the proteoglycan molecule is polydisperse in nature.

Tsiganos and Muir [4] have reported that proteoglycans may be aggregated with one another by means of glycoprotein linkage (Figs. 8.3 and 8.4).

A tiny part of a proteoglycan aggregate form cartilage matrix. The "core" proteins of each proteoglycan are attached to the hyaluronic acid backbone at intervals of 30–60 sugar dimmers (uronic acid plus amino

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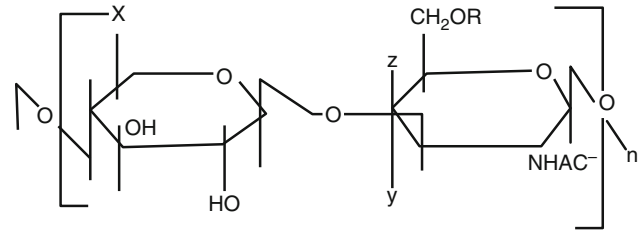


URONIC ACID
OR
NEUTRAL SUGAR

N-ACETYL-GLUCOSAMINE
OR
N-ACETYL-GALACTOSAMINE

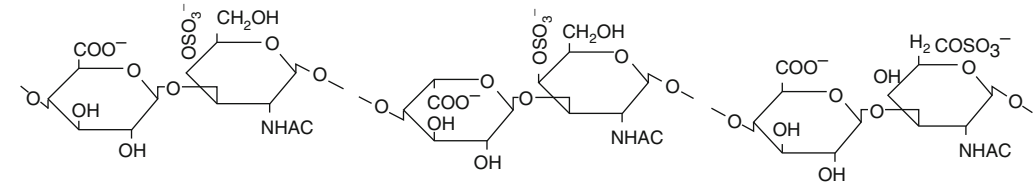
R = H OR SO_3^-

X = CH_2OH OR COO^-

Y = H OR OH OR OSO_3^-

Z = H OR OH OR OSO_3^-

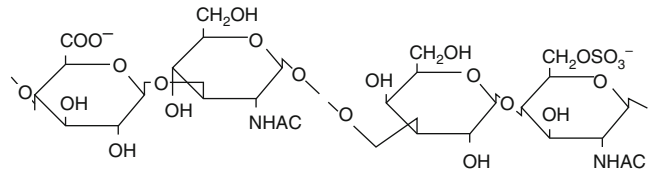
Fig. 8.1 General structure of mucopolysaccharides



CHONDROITIN SULPHATE A.

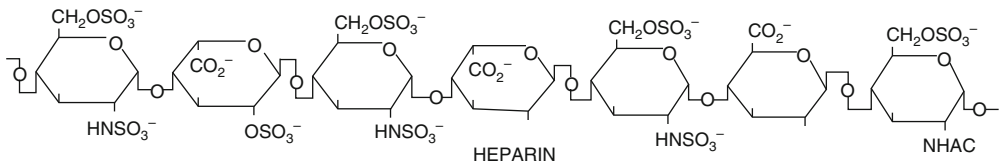
CHONDROITIN SULPHATE B.
OR
DERMATAN SULPHATE

CHONDROITIN SULPHATE C.



HYALURONIC ACID

KERATO SULPHATE



HEPARIN

Fig. 8.2 Structure of mucopolysaccharides

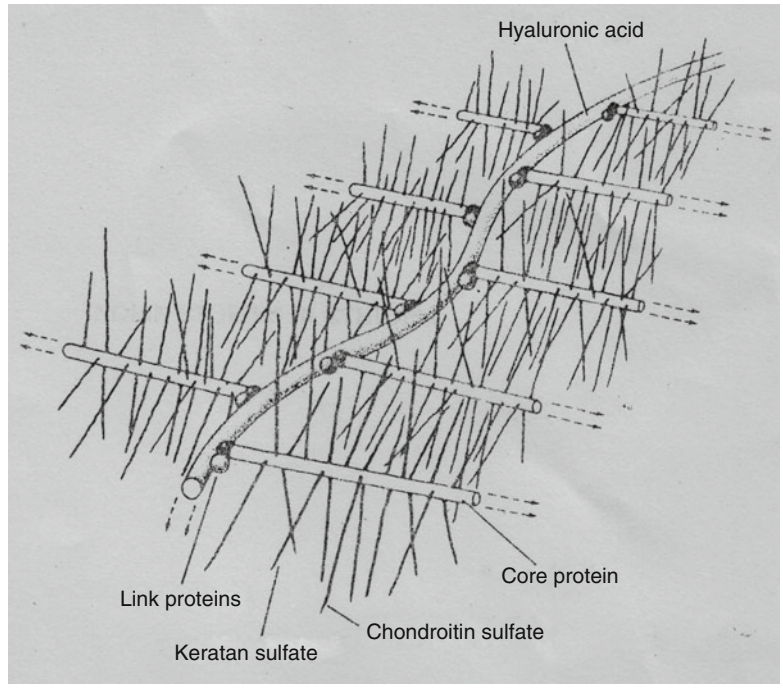


Fig. 8.3 Proteoglycal aggregate

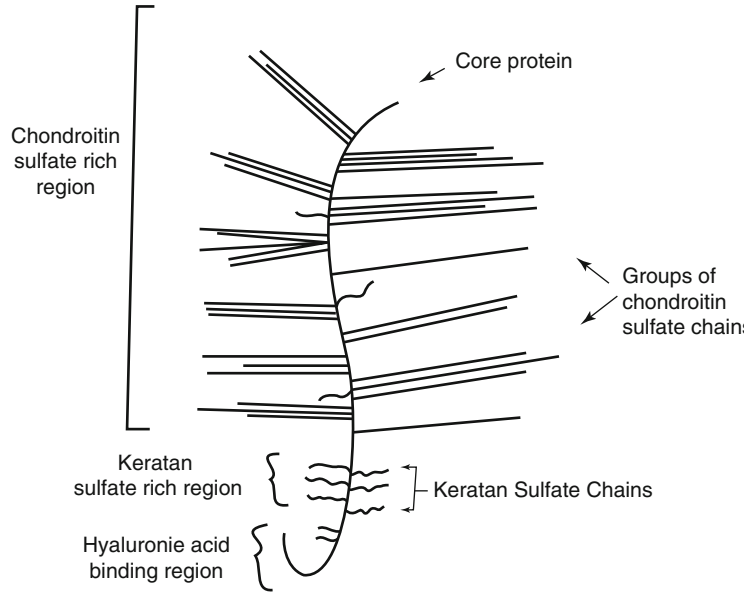


Fig. 8.4 Tentative model for the proteoglycan monomer structure

sugar) along the backbone. Linking proteins are found at the attachment sites. The GAG molecules (keratin sulfate and chondroitin sulfate) are attached to the core proteins; a given core protein molecule (molecular weight up to 2×10^5) may have 100 side-chains of chondroitin sulfate (molecular weight up to 2×10^4) and 30 to 60 sidechains of keratin sulfate (molecular weight up to 4 or 8×10^3), and so will be much larger and more complex than the drawing indicates. Since a hyaluronic acid backbone may vary between 4 and 0.4 microns in length, since the length of core proteins is highly variable, and since the length of GAG molecules on the core proteins is also variable and large, the total volume occupied by an aggregate is very great. And, of course, its potential variability in detailed structure is enormous [7, 8].

MPS or GAG is classified as neutral and acidic form. Neutral MPS contains no carboxglic (--COO^-) or Sulfate (--$SO_3^=$). These are present in blood group

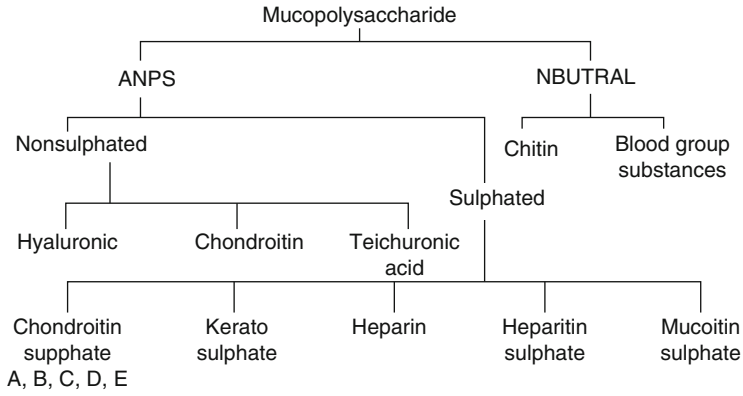


Fig. 8.5 Classification of mucopolysaccharide

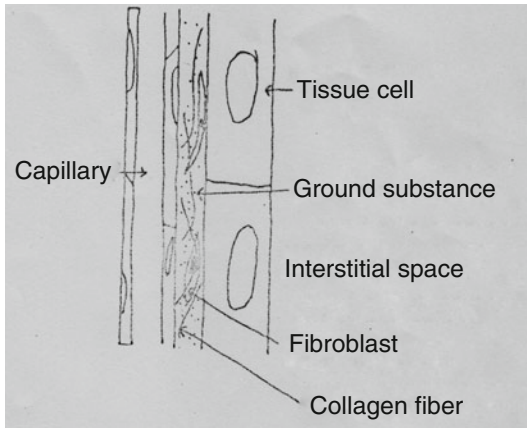


Fig. 8.6 Structure of connective tissue

substance. Acid MPS (AMPS) are nonsulfated and sulfated. Example of nonsulfated AMPS is hyaluronic Acid (Hy). and chondroitin, main sulphated AMPS are chondrotin sulfates (C-S-,A,B,C,D,E). Heparin and Heparan Sulphate (Fig. 8.5).

Matrix

Acidmucopolysaccaride (AMPS) in different connective tissues present mostly in intersticial space, which is known as Matrix (Fig. 8.6). The matrix consists of ground substance, fibres and impregnating materials. The Matrix contains AMPS, inorganic ions, water, organic compound in transit from the blood to the cell and from the cell to the blood and some macromolecules which have escaped from the Arteriolar end of capillaries. All the compounds and the ions are in some sort of association with the AMPS either directly or indirectly. Thus cations formed electrostatic bond with the polyanaion as also the water as Hydronium ions (H_3 O^+). The salts which are transported consist of the number of cations and anions. The other compound for e.g., Nutrients, intermediate compounds, waste products and probably the salts too are held in interstices of the gel formed by the union of the hydronium ion with the anion of the AMPS.

It may be assumed that a large quantity of extra cellular fluid is maintained by a large amount of polyelectrolyte, i.e., the AMPS molecule. These AMPS secondarily enhance the transport of cations which are indispensible for cellular activities mainly for synthetic process.

Function of Mucopolysaccharide

The presence of free carboxylic ($-Coo^-$) and sulphahte ($-SO_3^=$) group on each repeating unit of AMPS molecule confers a high negative (−) charge on it; such a highly charged polyelectrolyte, linear in configuration, interacts with water (H_3O^+) and cations (+), such as Na^+, Ca^{++}etc. This property of interaction between oppositely charged ions in AMPS is responsible for some of their biological functions. It seems likely that specific functions result from both the quantitative localization of AMPS as well as the specific chemical and physicochemical properties of the individual substances. It is clear from Grossman's finding that even very closely related AMPS may exhibit markedly different properties. The effect

of various hormones (relaxin, cortisone, testosterone) on certain tissues causes polymerization of the ground substance. Depolymerization of mucoprotein decreases the binding capacity of AMPS for cations. The binding of metals by monomers is less strong than by polymerized acids.

The AMPS fulfill diverse functions in the animal body. According to Dorfman et al. [9] the functions are as follows:

1. Supporting medium of any organ.
2. Water metabolism in connective tissue.
3. Control of electrolyte in connective tissue.
4. Organization of inorganic crystal or calcification.
5. Anticoagulant function of mucopolysaccharides.
6. Defensive function of mucopolysaccharides.
7. Wound healing.
8. Lubrication function.
9. Cleaning activity.
10. Maintance of stable transparent medium of the eye.
11. Interaction of MPS with collagen drug metabolite.

Among the above functions of AMPS we are interested in only water and electrolyte metabolism in fetuses and make correlation with AMPS concentration.

Total body water in an adult person constitutes 60 % of body weight; the water is distributed in the following way: 30–40 % is intracellular and 20–25 % is extracellular. In a new born child, however, the total body water is 78 % of body weight, 30–40 % is intracellular and nearly the same amount is extracellular. In fetuses around 6 months, the total body water is about 82 % of body weight; the intracellular water is again around 30–40 % and the extracellular water is either the same as or even more than the intracellular. Fetuses about 10 weeks of age contain 85 % of water where extracellular water is perhaps more than the intracellular. Extracellular water, therefore is highest in early fetal life and decreases as it grows older. The decrease of extracellular water relative to intracellular continues after birth and is most evident in the first few months of life. The tissues in the early periods of gestation are in a highly anabolic state, which of course, require a large amount of nutrient substances, which are supplied continuously through the blood stream. The excess extracellular water probably allows solution of large amount of such nutrients and enables the tissue cells to be supplied with the required compounds from the blood stream through the connective tissue spaces.

The negative charges of AMPS maintain an accumulation of water (H_3O^+) in the interstitial tissue space. Hyaluronate as a hydrated gel has an important function to bind water in the skin. As ageing progresses, there is a relative decrease of Hy and an increase of CS in skin [10]. According to Dorfman and Schiller [11], it is not easy to explain why tissue hydration is specifically connected only with Hy but not with sulphated MPS. Both fibroblast cells and mast cells play an important role in the homeostatic mechanism maintaining water in intercellular space.

The structural organization of water in the protein has its role in biological functions such as ionic cell selectivity [12]. Tropocollagen molecule holds much of CT water [13].

Electrolytes Control in the Connective Tissue

The ground substance of the CT contains all the transit compounds other than water between parenchymal cells and circulation. Among the transit compounds diffusible cations (Na^+, K^+, Ca^{++}, Mg^{++}) are mostly related to one or other of the ground substance colloid compounds, the negatively charged immobile aggregate of AMPS-protein complexes.

In tissues, the most common cations of extracelluar space are the sodium ions so that the polysaccharides occur in tissues almost entirely as sodium salts. K^+, Ca^{++} and Mg^{++} ions are also associated with polysaccharides.

The object of our work:

1. To determine the total water and extracellular water of the organs and electrolyte (Na^+,K^+) content in tissues and Serum at different gestation period.
2. To estimate the total AMPS content of certain human fetal organs in different gestation periods.

3. To fractionate the AMPS into the three usual fractions, i.e., hyaluronate, chondrotin sulphates and heparin in these organs.
4. To undertake some studies on the composition of these classes of AMPS form the organs.
5. To determine the major electrolyte concentrations of the organs and to correlate the quantity and quality of the AMPS to the water and the major electrolyte concentrations in these organs.
6. To measure the activity of the enzyme glucosamine-6-phosphate synthetase (Gm-6-p is the major precursor of AMPS) in different gestation periods.

Methods of Investigation

Fetuses were obtained by hysterotomy after obtaining required consent of mothers (from 1977) admitted for Medical Termination of Pregnancy (MTP). The fetuses were dissected at once in most of the cases. The organs were dissected in toto. After the determination of total weights of the organs aliquots were taken for respective measurements. The following organs were selected:- liver, lung and brain. The following estimations were carried out in aliquots of the above organs.

1. Total water and extracellular water and electrolyte content:

   Total water content was determined by weighing about 300 mg. of tissue in a preweighed tube and keeping it at 105 °C for 18 h. The difference in weight was taken to be due to evaporation of water.

   Extracellular water of tissues was determined from Na-thiocyanate space by the method of Eder [14]. Mothers were injected 10 ml. of Na-thiocyanate solution, 5.0 % (in sterile form) 4–6 h prior to operation. Na-thiocyanate concentration of fetal serum and tissues were determined.

   After the evaporation of water the tissue was ashed in a muffle furnace at 500 ± 25 °C for 4 h; after the furnace was cooled the tubes were taken out and when they came to room temperature, they were reweighed. The difference was taken to represent the ash content. The ash was dissolved in 5 ml. of 6-N HCL. The solution was centrifuged. Sodium and potassium concentrations were determined in appropriately diluted supernatant by flame photometry.
2. Acid Mucopolysaccharides (AMPS) in fetal tissues:

   Extraction of AMPS and fractionation were carried out according to Singh and Bachhawat [15], with slight modification. Bachhawat's method was based on the method of Dorfman [16] which was itself a modified method of Scott [17].

   Aliquotes of the tissues were dried with acetone and made lipid free with 2:1 Chloroform and Methanol mixture. AMPS was extracted from the defatted dry tissues with 0.1M acetate buffer, pH 5.0. Extracted AMPS was made protein free by papain digestion.

Fractionation of AMPS

Extracted AMPS was fractionated by the method of Schiller et al. [16]. Extracted AMPS is precipitated with Cetyl Pyridinium Bromide (CPB). The CPB-AMPS complex was collected along with celite. From this complex AMPS were fractionated to fractions I, II and III by different NaCl Concentration as 0.4M, 1.2M and 2.1M respectively. AMPS was precipitated by Alcohol, centrifuged and dissolved in a small volume of water. It was ready for constitutional analysis.

Hexuronic acid content was determined by Dische's carbozole method [18] as modified by Bitter and Muir [19] and further slight modification by Singh [15]. The Uronic acid content was multiplied by 2.4 to get an approximate value of total AMPS content.

Hexosamine content was estimated from various AMPS fractions. The fractions were hydrolyzed with concentrated HCl (to make the final conc. 0.6 N) at 100 °C. in the sealed tubes for 4 h. The hydrolysates were dried in a vaccum desiccators. The dried samples were dissolved in a

small volume of water and made ready for Hexosamine (Hm.) analysis. Determination of Hm is based on the Elson-Morgan [20] and Morgan-Elson [21] methods for free Hm and N-Ac-Hm respectively. Judoseig and Benmanan [22] used a method to determine Galm and total Hm content by measuring the color intensity in cold and hot reactions.

Sulphate estimation from total AMPS was estimated by $BaCl_2$ – gelatin method of Dodgson and Price [23].

Glucosamine-6-p Synthetase (E.C. 2.6.1.16)

It is the primary reaction in the Biosynthesis of AMPS. Assay of this Enzyme was done by the method of Ghosh et al. [24]. Liver is the main source of this enzyme. Fetal liver homagenate was incubated with substrate G-6-P and Glutamine in K-Phosphate buffer (0.2M, pH7.6). Glm-6-p was estimated by the method of Morgon and Elson [21]. Enzyme activity was expressed as µmole/mg tissue/h and Specific activity as µmole/mg protein/h. Soluble protein was estimated by the method of Lowry et al. [25].

Result

For the sake of convenience the fetuses have been divided into six groups – A, B, C, D, E and F according to a difference in the gestation period of 4 weeks. Table 8.1 shows the grouping of the 115 fetuses investigated in the present series of these fetuses; 15 belonged to group A, 26 to B, 36 to C, 21 to D, 14 to E and 3 to F. Three organs, Liver, Lungs and Brain, were selected for the study of AMPS; water electrolytes and the Key enzyme of AMPS Synthesis Glucozamine-6-p synthetase was also studied in the above three organs.

Table 8.1 Grouping of fetuses according to body weight and gestational age (week)

Group	No. of fetuses	Week	Weight g
A	15	8–12	1.5–14.5
B	26	13–16	15.6–108
C	36	17–20	115–295
D	21	21–24	330–660
E	14	25–28	715–1025
F	3	29–32	1,055–1,650

Table 8.2 Total mucopolysaccharieds of human fetal liver of different gestation period

Group	Age weeks	No. of fetuses	Total uronic acid mg/g. Dry defated tissue
A	8–12	–	ND
B	13–16	7	0.74 ± 0.16
C	17–20	10	0.65 ± 0.07
D	21–24	10	0.56 ± 0.07
E	25–28	5	0.55 ± 0.09
F	29–32	–	ND
Postmortem	0–8 days	6	0.35 ± 0.02

Liver

AMPS Concentration in Human Fetal Liver Tissues

The dehydrated defatted tissues were used for the determination of AMPS. Table 8.2 shows the total AMPS concentration in fetal tissues in different gestational periods. The result is expressed as Uronic acid per g of dry lipid free tissues. The uronic acid content of fetal liver varied from 0.5 to 0.7 mg/g, in postnatal life the amount was around 0.3 mg/g.

Mucopolysaccharides in human fetal liver and their fractions (Tables 8.3 and 8.4). In group A, the livers were too small to be analyzed. In group B, we could determine the total amount of UA in fraction 1. In groups B and C the total amount of fraction I was found to be the same; the hexosamine present was mostly glucosamine but the content of sulphate was almost equal to that of the UA. Thus this fraction contained the nonsulfated hyaluronic acid, some amount of heparitin sulfate and a small amount of CS. In groups D and E, total AMPS slightly decreased and this fraction also contained predominantly hyaluronic acid but considerable amount of chrondroitin and heparatin sulfate was found to be eluted in this fraction. In post mortem specimens of babies of 0–8 days ages, the total amount of fraction I was found to be much less than in the fetal specimens but the nature of the

Table 8.3 Mucopolysaccharides in human fetal liver and their fractions

Group	Age (wks.)	No. of cases	Total	Uronic acid	Mg/g dry defatted tissue Fractions	
				I	II	III
A	8–12	0	ND	ND	ND	ND
B	13–16	3	0.74 ± 0.16	0.34 ± 0.06	0.25 ± 0.03	0.08 ± 0.04
C	17–20	12	0.65 ± 0.07	0.40 ± 0.02	0.21 ± 0.02	0.04 ± 0.02
D	21–24	10	0.56 ± 0.07	0.30 ± 0.05	0.24 ± 0.04	0.03 ± 0.02
E	25–28	5	0.55 ± 0.09	0.38 ± 0.08	0.20 ± 0.07	0.07 ± 0.01
F	28–32	0	ND	ND	ND	ND
P.M	0–8 days	7	0.15	0.15	0.04	0.35 ± 0.02

Table 8.4 Composition of AMPS of fraction –I

Group	Age	No. of cases	Uronic acid mg/g dry defatted tissue	Hexosamine*	Glucosamine*	Galactosamine*	Sulfate*
A	8–12	0	ND	ND	ND	ND	ND
B	13–16	3	0.34 ± 0.06	ND	ND	ND	ND
C	17–20	12	0.40 ± 0.02	1.30	1.27	0.03	0.9
D	21–24	10	0.30 ± 0.05	1.25	1.02	0.23	1.0
E	25–28	5	0.38 ± 0.08	0.90	0.75	0.15	0.8
F	29–32	–	ND	ND	ND	ND	ND
P.m	0–8 days	7	0.15	0.75	0.60	0.15	1.37

*Expressed as molar ratio of Uronic Acid

Table 8.5 Composition of AMPS of fraction –II

Group	Age	No. of cases	Uronic acid mg/g dry defatted tissue	Hexosamine*	Glucosamine*	Galactosamine*	Sulfate*
A	8–12	0	–	ND	ND	ND	ND
B	13–16	3	0.26 ± 0.08	ND	ND	ND	ND
C	17–20	12	0.21 ± 0.02	0.97	0.13	0.84	0.87
D	21–24	10	0.24 ± 0.04	1.20	0.13	1.07	1.5
E	25–28	5	0.19 ± 0.07	0.86	0.38	0.48	0.98
F	29–32	–	–	ND	ND	ND	ND
P.m	0–8 days	7	0.16 ± 0.02	0.70	0.20	0.50	0.98

*Expressed as molar ratio of Uronic Acid

material eluted in this fraction was observed to be a mixture of HY and CS and heparitin sulfate.

Table 8.5 shows that in groups A and B, the fraction II was insufficient in amount for determination of various constituent. In group C, the amount of fraction II as determined by the total hexuronic acid content was higher than in the livers of babies dying after 0–8 days of life. When the fraction was, however analyzed for the glucosamine and galactosamine contents, most of the aminosugar was found to be galactosamine rather than glucosamine, which should be the preponderant aminosugar in this fraction.

In Table 8.6, Fraction III shows the content of heparin was low as compared to Fractions I and II but by and large the heparin content in fetal life was slightly greater in group B and E than in postmortem liver in postnatal life. This fraction also cannot be considered to consist of heparin only, as a considerable amount of galm was present although heparin contains Glm rather than Glam. The sulfate content was characteristic of heparin in groups D and E but low in group C and post-mortem.

Table 8.6 Composition of AMPS of fraction –III

Group	Age	No. of cases	Uronic acid mg/g dry defatted tissue	Hexosamine*	Glucosamine*	Galactosamine*	Sulfate*
A	8–12	0	–	ND	ND	ND	ND
B	12–16	3	0.08 ± 0.04	ND	ND	ND	ND
C	16–20	9	0.04 ± 0.02	0.88	0.44	0.44	1.0
D	20–24	8	0.02 ± 0.02	0.90	0.77	0.13	3.0
E	24–28	7	0.07 ± 0.02	0.71	0.68	0.03	3.1
F	28–32	–	–	ND	ND	ND	ND
P.m	0–8 days	6	0.04 ± 0.02	1.2	1.1	0.1	1.0

*Expressed as molar ratio of Uronic Acid

Table 8.7 Total water, ash, sodium and potassium contents of human fetal liver. (Values are expressed in wet tissue)

Group	Gest, period weeks	Water g%	Ash g%	K^+ mEq/kg	Na^+ mEq/kg
A	8–12(7)	80.7 ± 1.2	1.62 ± 0.93	41 ± 5	94 ± 10
B	13–16(10)	79.4 ± 0.5	1.90 ± 0.13	43 ± 4	71 ± 7
C	17–20(20)	78.5 ± 0.3	1.63 ± 0.02	51 ± 2	67 ± 8
D	21–24(11)	78.3 ± 0.6	1.68 ± 0.15	48 ± 5	60 ± 10
E	25–28(8)	77.6 ± 0.4	1.31 ± 0.18	44 ± 4	66 ± 10
	0–8 days(4)	78.2 ± 1.3	1.57 ± 0.19	44 ± 3	62 ± 3
	8 (adults)	69.0 ± 1.6	1.42 ± 0.07	57 ± 3	63 ± 12

Water and Electrolyte Content of Human Fetal Liver (Including Different Compartments of the Tissue)

Water content of human fetal livers at different periods of gestation is shown in Table 8.7. It varied from 80.5 % of the organ weight at 8–12 weeks (Gr. A) to 77.6 % at 25–28 weeks (Gr. E). The decrease was progressive except in the last two groups where the water content was almost unchanged. We could obtain livers of four children who died from prematurity and other causes; the water content in their livers was also like that of a fetus at 21–28 weeks (Gr. F), namely around 78 %. The water content of adult livers obtained by surgical biopsy was around 69 %. Water in the body was compartmentalized mainly into two compartments, the extra and intracellular compartments.

We studied the water and electrolyte in different compartments of tissues. Many compounds as per e.g., inuline, sucrose, NaSCN, radioactive Na^+, Cl, $SO_4^=$ etc. have been used for the determination of extracellular space. We selected NaSCN to determine extra cellular space. We injected 10 ml sodium thiocyanate solution (5 %) to the mother 4 h before the fetus was taken out. The fetus was put as usual, on ice at the operation theater, and immediately brought to the laboratory where it was dissected. Fetal blood was obtained by cardiac puncture into a heparinized syringe and the blood was immediately centrifuged to get the plasma. Plasma thiocyanate concentration was measured by the method described by Eder [14]. The tissues were homogenized in water (1 ml per g T) and then the method was followed exactly as described for the plasma.

Table 8.8 Thiocyate concentrations in body fluids (mg/dl)

Maternal serum	(9)	4.7 ± 0.91
Fetal serum	(9)	2.7 ± 0.21
Amniotic fluid	(3)	0.48, 0.22, 0.94
Fetal bladder fluid	(2)	0.81, 0.48
Fetal C.S.F.	(1)	0.44

Table 8.8 shows the concentration of thiocyanate in maternal serum was higher than the concentration in fetal serum. The amiotic fluid had lower thiocyanate concentration than fetal serum; the fetal bladder fluid similarly had lower concentration as also did the cerebrospinal fluid.

Since throcyanate was found to be permeable across the placenta and since we assumed that fetal circulation was greater than the adult and fetal urinary excretion slower than the adult, we presumed that thiocyanate had assumed equilibrium in the fetus by 4 h. We, therefore, took thiocyanate space concentration in fetal organs to represent extracellular space.

The extracellular space per 100 g of tissue was calculated. According to the following formula – Extracellular space = $x/y \times 100 \times 1.1$, where x = Thiocyanate concentration of tissue in mg% and y = thiocyanate, concentration of plasma in mg% and 1.1 is a constant for the Donnan Equilibrium factor. The total water per 100 g tissue was calculated by drying the tissues to constant weight at 105 °C (usually for 18 h). The intracellular water was taken to be the difference of total organ water and extracellular space. Results are shown in Table 8.9. The thiocyanate space varied from 53 ml per 100 g of liver at 16 weeks of gestation to 43 ml at 27 weeks. As the total water did not vary much during the different gestation period, the corresponding intracellular water (i.e., total water – thiocyanate space) increased from 27 ml per 100 g at 16 weeks to 35 ml at 27 weeks.

Table 8.9 Water content and distribution of human fetal liver

Body weight	Approx. gest. period	Total water g%	Extracellular g%	Intracellular g%
95	16	80	53	27
101	16	79	51	28
107	16	79	51	28
120	17	80	53	27
250	20	81	45	36
330	23	79	46	33
375	24	80	46	34
402	25	79	46	33
877	27	78	43	35

The metabolism of water, electrolytes and MPS is closely interrelated. The matrix containing the polyanionic AMPS is supposed to attract the hydronium inos (H_3O^+) and the common cations like sodium (Na^+) and potassium (K^+) ions.

The more charged it will be, the more it attracts the cations which again, in their turn, will bring the anions like the chloride and bicarbonate around them. We therefore, determined the sodium and potassium concentrations in the ashed materials of different organs including the liver. Results on the liver are shown in Table 8.7. Potassium concentration in mEq/kg of body weight varied from 41 to 51; virtually there was no change at different periods of gestation. Sodium content, of the liver, however, in earlier periods of gestation, e.g., in group A and B was higher than in later periods.

Correlation of Water, Electrolytes and AMPS Concentration

Fetal tissues contain a greater quantity of water than adult tissues. This extra water is chiefly extracellular in location. The thiocyanate space in adult liver varied from 30 % to 35 % whereas in fetal liver it was from 43 % to 53 %.

We measured only the sodium and potassium contents of the fetal and maternal plasma; the

Table 8.10 Sodium and potassium concentrations in human fetal liver (in different compartments). Valued are expressed in wet tissue

Gr	CNS spray	Intracellular water	Plasma Na^+	Plasma K^+	Extracellular liver Na^+	Extracellular liver K^+	Intracellular liver Na^+	Intracellular liver K^+
–	Ml/kg	Ml/kg	mEq/L	mEq/L	mEq/kg	mEq/kg	mEq/kg	mEq/kg
C	520	275	140	11.6	79.1	6.6	0.0	44.4
D	465	320	138	10.1	70.6	5.2	0.4	42.8
E	460	325	135	12.5	68.4	6.3	0.6	37.7
Adults	320	370	142	4.8	49.9	2.0	7.1	55.0

Intracellular Water = Total water – CNS space
Extracellular Na = Plasma Na X 1.1 (Donnan Eq) X CNS Space
Extracellular K = plasma K X 1.1 (Donnan Eq) X CNS Space
Intracellular Na = Total sodium – Extracellular sodium
Intracellular K = Total Potassium – Extracellular Potassium

Table 8.11 Mucopolysaccharides of the developing human lung and their Fractionations

Gest. Pd	No. of cases	Total	Uronic acid mg/g dry defatted tissue fraction		
			I	II	III
8–12	0	ND	ND	ND	ND
13–16	4	4.59 ± 0.49	3.26 ± 0.35	1.21 ± 0.16	0.13 ± 0.04
17–20	7	2.94 ± 0.24	1.79 ± 0.12	0.96 ± 0.07	0.19 ± 0.09
21–24	3	2.42 ± 0.28	1.67 ± 0.47	0.65 ± 0.21	1.12 ± 0.019
25–28	5	2.21 ± 0.11	1.26 ± 0.08	0.85 ± 0.13	0.09 ± 0.02
28–32	0	ND	ND	ND	ND
0–8 days	6	0.70 ± 0.14	0.23 ± 0.06	0.39 ± 0.09	0.08 ± 0.02

total cations (Na^+, k^+) in fetal plasma was 151.6 mEq/L in the serum and 146.8 mEq/L in the mother (Table 8.7). The difference was statistically significant ($p < 0.02$). A greater difference which may have some role to play in the amount of the extracellular fluid in the tissues space in the fetal life lies in the hyaluronate and total AMPS contents of the fetal organs.

The thiocyanate concentration in fetal plasma was less than the thiocyanate concentration in maternal plasma. The elaboration of fetal urine is negligible and the thiocyanate concentration in bladder fluid was also much lower than in the plasma. We should have expected the thiocyanate concentration in fetal plasma to be higher than the maternal plasma if the compound was continually being filtered through the placenta. Perhaps, only the maternal thiocyanate which was bound to the plasma proteins actually filtered through the placenta and the free fetal thiocyanate might pass in the reverse direction. An equilibrium may probably be attained which ultimately resulted in fetal thiocyanate concentration being lower than the maternal.

Table 8.10 notes that the electrolyte concentration in the different compartments of the fetus was apparently quite distinct from those of an adult. Sodium contents of the extracellular fluid in fetal liver were greater than in adult liver. Since the total thiocyanate space was also bigger in the fetal liver, the extracellular space in fetal liver contained as much as twice the amount of sodium per kg of organ weight as in adult liver. The potassium concentrations as well as contents in the total thiocyanate space of the fetal livers were higher than those of the adult livers. Potassium concentration per litre of intracellular fluid was found to be 152 mEq in adult liver, whereas in fetal livers it progressively decreased form 161 mEq/L in fetuses of 17–20 weeks gestation to 116 mEq/L in 25–28 weeks of gestation.

Lung

Total AMPS of Lung and Its Fractionations

Total AMPS content of lung was estimated as total uronic acid content in the CPB precipitated material of the proteolyzed extract and expressed as mg per g of dry defatted tissue (Table 8.11). The lung is a soft tissue as compared with the liver. The process of extraction of AMPS from lung tissue was exactly the same as from the liver.

It is apparent from the Table 8.11 that the total AMPS content of the fetal lung was higher in fetuses of early gestation period than in those of the later periods. When compared to the liver (Table 8.11), the AMPS content of the lung was four to six times higher in fetuses of corresponding gestational age. With growth of the lung the amount of AMPS per g of tissue gradually and progressively decreased until at 29–32 weeks, the amount of AMPS was about a half of what was found in fetuses of 12–16 weeks. However, in all these ages the uronic acid content of the lung was about four to six times that of the liver.

Different Fractions of Total AMPS of Human Fetal Lung and Its Composition

Aliquots of each fraction were tested for uronic acid, total hexosamine, galactosamine, glucosamine and sulphate Results were expressed as molar ratio of uronic acid.

Table 8.12 Composition of different fractions of AMPS. Composition of mucopolysaccharides of human fetal lung (fraction I)

Group	Week age	No. of cases	Total Ua mg/g of dry defatted tissue	Hexosamine[a]	Glucosamine[a]	Galactosamine[a]	Sulphate[a]
A	8–12	–	ND	ND	ND	ND	ND
B	13–16	4	3.26 ± 0.04	ND	ND	ND	ND
C	17–20	7	1.79 ± 0.12	0.94	0.90	0.04	0.17
D	21–24	3	1.66 ± 0.47	0.79	0.78	0.01	0.20
E	25–28	5	1.26 ± 0.08	0.81	0.65	0.16	0.29
F	29–32	–	ND	ND	ND	ND	ND
P.M.	0–8 days	6	0.23 ± 0.66	1.2	0.90	0.13	0.55

ND Not Done

[a]Expressed as molar ration of Uronic acid

Table 8.13 Composition of mucopolysaccharides of human fetal lung (fraction II)

Group	Week age	No. of cases	Total Ua mg/g of dry defatted tissue	Hexosamine[a]	Glucosamine[a]	Galactosamine[a]	Sulphate[a]
A	8–12	–	ND	ND	ND	ND	ND
B	13–16	4	1.22 ± 0.16	ND	ND	ND	ND
C	17–20	7	0.96 ± 0.07	0.87	0.20	0.67	0.73
D	21–24	3	0.64 ± 0.21	0.82	0.20	0.62	0.87
E	25–28	5	0.86 ± 0.13	0.73	0.17	0.56	0.86
F	29–32	–	ND	ND	ND	ND	ND
P.M.	0–8 days	6	0.39 ± 0.09	0.74	0.03	0.71	1.01

ND Not Done

[a]Expressed as molar ration of Uronic acid

Table 8.14 Composition of mucopolysaccharides of human fetal lung (fraction III)

Group	Week age	No. Of cases	Total Ua mg/g of dry defatted tissue	Hexosamine[a]	Glucosamine[a]	Galactosamine[a]	Sulphate[a]
A	8–12	–	ND	ND	ND	ND	ND
B	13–16	4	0.13 ± 0.04	ND	ND	ND	ND
C	17–20	7	0.19 ± 0.09	0.67	ND	ND	0.65
D	21–24	3	0.12 ± 0.02	0.62	0.42	0.20	2.1
E	25–28	5	0.09 ± 0.02	0.67	0.41	0.46	2.5
F	29–32	–	ND	ND	ND	ND	ND
P.M.	0–8 days	6	0.082 ± 0.02	1.1	1.0	0.1	0.8

ND Not Done

[a]Expressed as molar ration of Uronic acid

Table 8.12 shows that fraction I predominantly consisted of hyaluronic acid. The hexosamine content was similar to the uronic acid content. Galm was virtually absent; almost all of the hexosamine was constituted of glucosamine, indicating all are Hyaluronic acid. However, there was not considerable amount of SO_4 in this fraction.

Table 8.13 shows that fraction II predominantly consisted of chondroitin sulphate. Of the total hexosamine, galactosamine constituted about two-third and glucosamine one-third; sulphate contents were present in almost 1:1 ratio to the uronic acid.

Table 8.14 shows that fraction III mainly consisted of heparin and as it contained glucosamine with N-sulphation and variable sulfation of four and six hydroxy groups of glucosamine, fraction III contributed only 3–4 % of the total AMPS. Throughout the period of gestation the

Table 8.15 Sodium and potassium concentrations of human fetal lungs (walues are expressed in wet weight)

Period of gest. weeks	Water g%	Ash g%	K^+ mEq/kg	Na^+ mEq/kg
9–12(5)	91.2±0.8	1.80±0.11	31±6.1	130±12
13–16(15)	89.0±0.3	1.50±0.10	30±2.5	128±18
17–20(20)	88.0±0.3	1.30±0.10	33±3.7	104±6
21–24(12)	86.6±0.3	1.25±0.07	34±3.2	90±16
25–28(9)	85.5±0.8	1.05±0.19	32±1.5	97±8
29–32(4)	82	1.2	25	110
0–8 days(5)	83	1.2	25	100
(8)	70±1.5	1.6±0.08	55±1.3	60±11

Table 8.16a Extra and intracellular water in lung

Gr.	Gest. per weeks	Water ml/kg	CNS space ml/kg	Intracellular water ml/kg
C	17–20(4)	880	570	380
D	21–24(4)	860	480	380
E	25–28(4)	850	452	398
Adult	(2)	690	325	365

Table 8.16b Sodium and potassium concentrations in different compartments

Gest. per weeks	Plasma Na^+ mEq/L	Plasma K^+ mEq/L	Extracellular lung Na^+ mEq/kg	Extracellular lung K^+ mEq/kg	Intracellular lung Na^+ mEq/kg	Intracellular lung K^+ mEq/kg
17–20(4)	137	13.7	85.9	8.5	18.1	22.5
21–24(4)	141	12.9	74.4	6.8	15.6	24.3
25–28(4)	145	12.0	72.1	5.9	24.9	25.3
(2)	133	3.8	47.5	1.5	12.5	53.5

content remained more or less the same. The hexosamine to uronic acid ratio was close to 1. The sulphate to uronic acid ratio of 2–2.5 is in conformity with the higher sulfation of heparin; but in early age of fetuses and in postmortem materials, the sulfate content was not that high.

Water and Electrolyte Contents of Lung

Water, ash, total potassium and total sodium contents of human fetal lung as found are shown in Tables 8.15, 8.16a, and 8.16b. Among human fetal organs, lungs were found to contain water in quantities more than any other organ except the brain. The water content gradually diminished, the ash content was more or less the same. The total potassium contents did not change very much with increasing age of the fetus and were lower than in the adult lungs. The total sodium contents were, however, very high at the early period of gestation and came down somewhat as the age of the fetus increased but even at 32 weeks were considerably higher than in the adult lung. The thiocyanate space in a fetus of earlier gestation period was very higher and came down as the age of the fetus increased but the intracellular space was more or less constant. In the human adult, the cation of the extracellular space was sodium. In the intracellular fluid of the fetus, however, sodium and potassium was calculated to be almost equal in amount. In the adult lung, the major intracellular cations was the potassium ion but some sodium was calculated to be present in the intracellular fluid also. Compared to the fetal lung, the adult lung contained much less mucopolysaccharides and extracellular sodium and potassium.

Brain

Mucopolysaccharides of the Human Fetal Brain at Different Gestation Periods

The amount of AMPS did not change with progress of gestation (Table 8.17). This is in contrast

Table 8.17 AMPS in fetal brain and their fractions

Age	No of cases	Total	Uronic acid mg/g defatted tissues		
			I	II	III
8–12(3)	3	1.88 ± 0.20	0.80 ± 0.20	0.77 ± 0.20	0.31 ± 0.04
13–16(5)	3	2.78 ± 0.10	1.75 ± 0.12	0.83 ± 0.14	0.20 ± 0.02
17–20(6)	6	2.39 ± 0.11	1.4 ± 0.26	0.76 ± 0.06	0.23 ± 0.12
21–24(3)	3	2.38 ± 0.06	1.53 ± 0.30	0.74 ± 0.04	0.11 ± 0.02
25–28(6)	4	2.5 ± 0.02	1.62 ± 0.05	0.80 ± 0.09	0.12 ± 0.02
P.m(3)	3	0.72 ± 0.06	0.17 ± 0.04	0.47 ± 0.10	0.08 ± 0.03

Table 8.18 Composition of mucopolysaccharides of Human Fetal Brain(Fraction I)

Age (Wk)	Uronic acid mg/g of dry defatted tissue	Hexosamine[a]	Glucosamine[a]	Galactosamine[a]	Sulphate[a]
8–12(3)	0.80 ± 0.20	–	–	–	–
13–16(5)	1.75 ± 0.12	1.01	0.95	0.05	0.23
17–20(6)	1.4 ± 0.26	1.01	0.90	0.11	0.53
21–24(3)	1.53 ± 0.30	1.05	1.00	0.05	0.61
25–28(6)	1.62 ± 0.05	1.1	1.00	0.10	0.50
P.m(3)	0.17 ± 0.04	1.0	0.92	0.08	1.05

[a]Expressed as molar ration of uronic acid. (Mean and standard deviation)

Table 8.19 Composition of mucopolysaccharides of human fetal brain (fraction II)

Age (Wk)	Uronic acid mg/g of dry defatted tissue	Hexosamine[a]	Glucosamine[a]	Galactosamine[a]	Sulphate[a]
8–12(3)	0.77 ± 0.20	–	–	–	–
13–16(5)	0.83 ± 0.14	1.5	–	–	–
17–20(6)	0.76 ± 0.06	1.1	0.30	0.80	1.65
21–24(3)	0.74 ± 0.04	0.90	0.35	0.55	1.12
25–28(6)	0.80 ± 0.09	0.85	0.31	0.54	1.0
P.m(3)	0.47 ± 0.10	0.97	0.07	0.9	1.1

[a]Expressed as molar ration of uronic acid. (Mean and standard deviation)

to the situation in the lung where the total AMPS underwent progressive decrease with increase in gestation period; in this respect the results were similar to the findings in the fetal liver although the amount of AMPS in the fetal liver was lower than that of the brain. In postmortem materials on neonatal brain the amount of AMPS was found to be in lesser amount than in fetal brains. When the AMPS of the fetal brain were fractionated, it was again found that fraction I was in a light preponderance, Tables 8.18, 8.19, and 8.20, show that hyluronate constituted a slightly greater fraction of the polysaccharides than the other two fractions. Table 8.18 shows that the hexosamine was mostly glucosamine but again there was considerable quantity of sulfates in this fraction. The high hyaluronate was a characteristic of fetal brain since postmortem specimens had a much lower hyaluronate and a relatively higher chondroitin sulphate content (Tables 8.18 and 8.19). Fraction II (Table 8.19) contained the chondroitin sulfates and heparitin sulphate; the predominant sugar was galactosamine although some glucosamine was found to be present in this fraction indicating the presence of heparitin sulfate. Fraction III (Table 8.20) constituted a smaller fraction of the AMPS as in the other organs. The molar ration of sulphate to uronic acid was more

Table 8.20 Composition of mucopolysaccharides of human fetal brain (fraction III)

Age (Wk)	Uronic acid mg/g of dry defatted tissue	Hexosamine[a]	Glucosamine[a]	Galactosamine[a]	Sulphate[a]
8–12(3)	0.31 ± 0.04	–	–	–	–
13–16(5)	0.20 ± 0.02	0.6	–	–	1.4
17–20(6)	0.23 ± 0.12	0.6	–	–	1.4
21–24(3)	0.11 ± 0.02	0.85	0.71	0.14	1.5
25–28(6)	0.12 ± 0.02	0.89	0.34	0.55	1.2
P.m(3)	0.08 ± 0.03	0.3	–	–	0.8

[a]Expressed as molar ration of uronic acid. (Mean and standard deviation)

Table 8.21 Water, sodium and potassium contents in human fetal brain (per 100 g wet weight)

Gest. period(wk)	Water g%	Ash %	K^+ mEq/kg	Na^+ mEq/kg
9–12(7)	91 ± 0.5	1.46 ± 0.06	40 ± 4	89 ± 3
13–16(12)	90 ± 0.9	1.26 ± 0.17	41 ± 2	98 ± 5
17–20(21)	90 ± 0.2	1.22 ± 0.06	48 ± 2	100 ± 2
21–24(10)	89.5 ± 0.2	1.19 ± 0.20	39 ± 3	78 ± 6
25–28(9)	88.2 ± 0.2	0.99 ± 0.06	36 ± 2	76 ± 10
0–8days(4)	81.7	1.25	58 ± 2	70 ± 5
(4)	81.5	1.15	64 ± 3	72 ± 4

Table 8.22 Sodium and potassium concentration in different fluid compartments of human fetal brain

Ges. Wk	Water ml/kg	CNS space ml/kg	ICW ml/kg	EC Na^+ mEq/kg	EC K^+ mEq/kg	IC Na^+ mEq/kg	IC K^+ mEq/kg
17–20(4)	902	460	442	69.0	6.6	31.0	41.4
21–24(4)	905	430	475	66.7	6.7	11.7	32.3
25–28(4)	910	460	450	71.3	5.9	4.7	30.1

No adult brain could be analyzed

than 1 in fraction III as it should be (Tables 8.17, 8.18, 8.19, and 8.20.

Sodium and Potassium Concentrations in the Human Fetal Brain

The water content of the brain varied form 91 % at 9–12 weeks to 86 % at 32 weeks. The potassium content of whole human fetal brain was between 36 and 48 mEq/kg.; in adult brains the potassium content was much higher (64 mEq/kg). The sodium content of the fetal brain varied from 76 to 100 mEq/kg. The sodium content of adult brain was found to be around 72 mEq/kg. The difference between the fetal and adult brain was very great indeed with regard to the sodium content (Table 8.21).

The thiocyanate space was determined in four fetuses each of groups C, D and E. Results are shown in Table 8.22. The total water and thiocyanate space did not change very much in the three groups. The extracellular sodium and potassium did not change. But there was a great reduction in the intracellular sodium content. In fetuses of earlier gestation period, i.e., 17–20 weeks, a large amount of intracellular sodium was present (Table 8.22).

Glucosamine-6-Phosphate Synthetase (EC 2.6.1.16) in Liver, Brain and Lung

Table 8.23 shows the activity of Gm-6-P synthetase of human fetal liver at different gestation periods upto 28 weeks. Results are compared with the activity of adult human liver obtained during cholecystectomy. The specific activity of fetal liver varied from 63 to 74 units (uM/g protein/h) at different gestation periods. These activities were 2.5–2.8 times that of human adult

Table 8.23 Glucosamine-6-phosphate synthetase in liver, brain and lung. (Enzyme activity in tissue)

Age in weeks	No. cases	μM product/g tissue/h		
		Liver	Lung	Brain
8–12	6	5.58 ± 0.75	ND	0.26 ± 0.01
13–16	7	3.10 ± 0.39	0.27 ± 0.07	0.27 ± 0.02
17–20	11	2.89 ± 0.24	0.23 ± 0.02	0.17 ± 0.01
21–24	6	2.90 ± 0.27	0.17 ± 0.02	0.17 ± 0.02
25–28	ND	ND	ND	ND
29–32	ND	ND	ND	ND
35–55 years	5	1.72 ± 0.24	0.50 ± 0.10	ND

Table 8.24 Glucosamine-6-phosphate synthetase in liver, brain and lung. (Specific activity of the enzyme)

Age in weeks.	No. cases	μM product/g protein/h		
		Liver	Lung	Brain
8–12	6	65.0 ± 4.0	ND	10.5 ± 1.6
13–16	7	68.0 ± 7.3	12.0 ± 2.3	11.5 ± 2.5
17–20	11	73.9 ± 3.4	11.5 ± 1.5	10.5 ± 0.7
21–24	6	68.0 ± 5.1	8.8 ± 1.1	14.0 ± 0.7
25–28	ND	ND	ND	ND
29–32	ND	ND	ND	ND
35–55 years	5	27.0 ± 5.1	9.4 ± 2.0	ND

liver. The difference between the activity of fetal liver and adult was statistically significant ($p < 0.001$). Ghosh et al. [24] showed the activity in 18,000 g supernatant in rat liver to be 20.2 μM/h/g protein which is also similar to human adult value. Spiro [26] from specific activity curve showed that the liver is the primary site for the synthesis of serum glucosamine. Phelp [27] purified and studied the kinetic property of rat liver enzyme.

The activity of the fetal lung was one-fifth to one-eighth of the activity of the corresponding fetal livers and the adult lung obtained in pneumonectomy specimen away from the site of lesions had an activity which was about a third of the adult liver. Enzyme activity is measured as the formation of product (Glucosamine-6-p) as μM/g tissue/h and Specific activity as μM/g protein/h (Tables 8.23 and 8.24).

Glucosamine-6-phosphate synthetase activity of the human fetal cerebral cortex is shown in Table 8.23, from 8 weeks of gestation to 24 weeks the activity varied from 10.5 to 14 units/g protein/h. The activity was compared to that of the lungs at corresponding gestation. Values are more or less similar. The enzyme activity of liver was six times higher than that of brain.

The effect of various hormones on certain tissues (relaxin, cortisone, testosterone) causes polymerization of the ground substance. It also increases the proportion of the soluble fraction of colloids compensated by the decrease of bound electrolytes arising from the increased binding strength of water. Depolymerisation of the mucroproteins decreases the binding capacity of MPS for cations. The binding of metal by monomers is less strong than by polymerized acids.

Discussion

MPS and Relationship with Water and Electrolytes

The amount of hexuronic acid in the extracted, partially purified MPS material is, therefore, a measure of the total MPS of an organ. In embryos the mesenchyme is principally cellular but in fetuses the matrix is quantitatively greater than the actual cellular element. The higher uronic

acid in fetal organs of early gestation period may be due to a relatively greater content of mesenchymal cells in a given volume of tissue. It must however, be cautioned that these cells are relatively undifferentiated and less ready to extrude the proteoglycans in the interstitial space.

Liver

Mucopolysaccharide

Fraction I, comprising Hy should not contain Glam and sulfate. Although glam content was proportionately low, there was quite a large amount of sulfate. It appeared, therefore, that some amount of Cs and Hs was present in this fraction. There was a diminution in the amount of fraction I, at later periods of gestation and in immediate postnatal life. Fraction II was also not a pure class of compounds in as much as in this fraction a large quantity of glucosamine was found. The sulfate content more or less corresponded with an equimolar quantity of hexosamine. The contamination might therefore, be either due to heparitin sulfate or even to heparin. The quantity was almost half of that found in Fraction I. Fraction III constituted less than 10 % of the total UA content and the fraction also contained a mixture of a variety of compounds. If fraction III constituted entirely of heparin, it should have a hexuronic acid ratio to sulfate more than 2. However in the earlier period of gestation and in adult livers this ratio was less than 2. It is only in the later periods of second trimester (21–28 weeks) that the ratio corresponded to that of heparin. During the progress of gestation there was a decrease in the total UA content of the liver; the decrease was mostly in the Hy fraction. We do not know the contribution of the individual proteoglycans to the overall function of the MPS. The decrease in Hy fraction with presumably a greater stability of the matrix specially in extrauterine life would go against the indispensability of Hy to form a stable structure with link proteins for the other polysaccharides.

Water and Electrolytes

The main difference in the sodium and potassium concentrations of the serum in fetal and adult sera consisted in the higher serum potassium content in fetal life. Whereas adult serum potassium concentrations were 4.8 mEq/L ±0.5, the corresponding concentrations in fetal sera were 10.8 mEq/L ±1.3. Since there was no difference in the sodium contents of fetal and adult sera, the total cation concentration and the total osmolarity of fetal sera were higher than those of adult sera. This higher osmolarity of fetal water probably has something to do with the floatation characteristic of the fetus in the amiotic fluid. The density of a few smaller fetuses was found to be 1.103 by water displacement method, whereas that of the amniotic fluid was around 1.117. The maintenance of this density was a function of the osmotic tension of the body fluids of the fetus. A teleological explanation of the higher serum potassium in fetal life may also be given. A growing organ actively synthesizing protein and depositing the protein in the tissues needs potassium. The higher serum potassium level in fetal life ensures a liberal supply. Furthermore, potassium concentration in the intracellular fluid of the fetal liver was lower than that of the adult liver. Perhaps, the sodium pump in the tissue is not as effective in fetal liver as in adult liver so that potassium leaks out into the extracellular fluid.

The thiocyanate space in fetal liver was greater than in adult liver. The extracellular fluid was therefore present in greater quantity in fetal life than in adult liver. In fact, the higher total water content of fetal liver may be ascribed entirely to the greater quantity of extracellular fluid. The intracellular fluid was not increased in fetuses. This huge amount of extracellular fluid is not present as free water but must be in a soluble form in combination with a macromolecule. The macromolecule is not presumably a protein but a polysaccharide, a mucoploysaccharide to be exact. Total mucopolysaccharide contents was higher in earlier gestation period than in later. The decrease was still more manifest in neonatal livers on postmortem examination. The total water content of fetal liver was lower than that of the adult liver. However, the decrease in total mucopolysaccharides from fetal to neonatal liver was more than the decrease in total water from fetal to adult life. It might therefore, be assumed

that amount the various functions of the mucopolysaccharides, only one function is related to its binding property with water. There are other important functions of this class of compounds, which are perhaps, more important in fetal life. The decrease of mucopolysaccharides during progress of gestation was almost entirely ascribable to a decrease in hyaluronates. Since the binding of hyaluronate to water is a well-known phenomenon, the relation between the decrease of hyaluronate content and water is more than coincidental.

The presence of a higher amount of water in the extracellular space necessitates retention of salts to maintain osmotic equilibrium. The total cation content of the extracellular fluid per kg of organ weight in fetal liver amounted to 75 mEq whereas in the adult liver it was only 52 mEq. The increase of extracellular water in fetal liver as compared to adult liver was between 50 % and 70 %. Therefore, the total cation content in extracellular fluid of fetal liver was increased relatively as well as absolutely. The total mucopolysaccharide contents of fetal liver in earlier gestation periods was about double that of neonatal livers. Hence the mucopolysaccharides must play an important role in the greater binding of not only water but of cations as well.

Lung

Mucopolysaccharide

Total MPS of the fetal lung were in all instances higher than those of the liver at the corresponding periods of gestation. It might signify a greater extracellular material in the fetal lung than in the fetal liver. However, as already mentioned, all of the acetone extracted, papain digested material of the fetal lung went into solution in the dilute alkali; but a fraction of the corresponding part of the fetal liver did not. It might be interpreted to mean that the fetal liver contained more differentiated fibrous elements than the lung counterpart. With progress of gestation there was decrease in total MPS per gm. of dry lipid free tissue in the lung as in the liver. These findings are in contrast with the observations of Horwiz and Crystal [28] on rabbit lung. They observed the GAG content to be relatively constant around 3.4 uM/g of dry weight in fetal lungs and to increase to around 6 uM/g in adult lungs. In human fetuses on the other hand the GAG content were found to be much higher (around 24 uM/g dry weight in the early period of gestation) than in rabbit lung and during growth of the lung there was marked decrease in the MPS content. The adult human lung was found to have a much smaller MPS content (around 3.5 uM/g. dry weight). The decrease might signify that the amount of matrix becomes less as the organ becomes more compact or the more differentiated a tissue becomes the less amount of extracellular space it holds. The decrease of the amount of uronic acid with the onset of extrauterine respiration is real and probably results from compression of the surrounding lung parenchyma by the expanding alveoli and absorption of intra alveolar and perialveolar material. The decrease of MPS with increase in gestation period may also be related to the changing populations of cell types found in the rat lung in the immediate neonatal period (Kaufman, Burris [29] and Wiebel, 1975). When the MPS was fractionated into Fraction 1, containing predominantly hyaluronic acid it was found that there was a pronounced reduction in this fraction with progress of gestation and especially after both, i.e., after extrauterine respiration has occurred. Fraction 2 containing mainly CS was also found to be highest at 14–16 weeks of gestation but the decrease with increasing period of gestation was not spectacular like Fraction 1. Again after respiration had set in, fraction 2 was also considerably reduced. Fraction 3 containing mainly heparin constituted 3–4 % of the total mucopolysaccharides in the fetal lung; in the neonatal lung this fraction was about 10 % of the total mucopolysaccharides. The heparin content of human fetal lung was therefore lower than that of the neonatal lung.

Water and Electrolytes

Among human fetal organs, lung was found to contain more water than any other organ except the brain. Thus at 9–12 weeks of gestation the water content of the lung was 91 %, while that of the liver was 80.5 %. The MPS content of the

fetal lung was also very high as compared to the liver. Thus at 13–16 weeks of gestation the total uronic acid per g of dry defatted lung was 4.6 mg, while that of the liver was only 0.74 mg. It may be that the greater amount of water in the fetal lung of earlier gestation period could be accounted for by its association as hydronium ions to the polyanions of the AMPS especially with Hy in extracellular fluid. The higher water content of the fetal lung at earlier gestation period could not be due to lesser cellularity at this period as the amount of DNA per gm. of tissue was found to be highest (unpublished doctoral dissertation of 1st author, Dr. Chameli Ganguly, 1978, Calcutta University, available at Calcutta University) in the earliest gestation period studied. As in the fetal liver the thiocyanate space was highest at the earliest gestation period and gradually come down. The intracellular water did not change much with increase in gestation period. Hence, the higher water content could be ascribed to the extracellular fluid only. The high amount of water in the extracellular space naturally would necessitate movement of salts in order to keep proper osmotic equilibrium. The total of sodium and potassium ions in the tissue, as a whole, underwent a progressive diminution from about 160 mEq at 9–12 weeks of gestation to 130 mEq at 25–28 weeks. In adult lungs, however, total sodium content was lower and total potassium higher than in fetal lungs. The distribution of these ions in the extra and intracellular fluid was also different in the fetal and adult lungs. The total sodium and potassium ions in the extracellular fluid of the fetal lung was much higher than that of the adult lung; the potassium ions in the intra-cellular fluid was correspondingly lower in the fetal lung than in the adult lung. Although the major cation of the extracellular space in fetal lung was calculated to consist of sodium, surprisingly in the intacellular fluid, a little less than half of the major cation was found to be sodium was calculated to be present in the intracellular fluid. The reason for this is unclear the estimation could not be wrong. We checked and rechecked in all possible ways. We sent some dried specimens abroad. Their values were similar with our.

Brain

Mucopolysaccharide

The MPS of the rat brain during development have been studied by Margolis et al. [30] and by others. We found that the total MPS were around 2 mg uronic acid per g of dry defatted tissue in human fetal brain at 8–28 weeks of gestation. The total MPS did not change significantly from 9 to 28 weeks of gestation. The water content in these brains, however, decreased progressively from 91 % at 9–12 weeks to 88.2 % at 25–28 weeks. Thus the water content could not be related to the total MPS content. The nonvariability of the total MPS at 9–28 weeks of gestation is also reflected in the hyaluronate (Fraction I) and chondroitin sulfate (Fraction II) contents; they also did not register much change. In neonatal postmortem brains both total MPS and hyaluronate contents (Fraction I) were much lower than in the fetal brain. Fraction II (chondroitin sulphate) was relatively predominant in the postmortem specimens. There was a reduction in Fraction III as gestation period increased. In previous studies on developing rat brain, a high amount of hyaluronic acid in early gestation was thought to be responsible for greater retention of water [30]; since the polymeric chains of hyaluronic acid were shown to have significant role in hydration and solvent transfer in tissues [31]. However, in none of the studies significant attention has been paid to Fraction III, containing heparin. Our findings that Fraction III in fetal brain underwent a decrease with progress of gestation may have something to do with the interstices of the brain mass permitting easier migration of neuronal cells, at earlier periods of gestation. The degree of polymerization of the polysaccharide units in Fraction III may determine the electrostatic holding of H_3O ions in the interstices of the neuronal and possibly glial cells too.

Water and Electrolytes

Brain is the most watery of all fetal organs; at very early periods of gestation, around 9 weeks, brain and lungs have water contents of around 90 % of the wet weight. This huge amount of

water in the brain is largely extracellular as judged by the thiocyanate space. Our finding of brain thiocyanate space in fetal brain of 46 % is not very disproportionate. Tissue spaces available to thiocyanate are higher than inulin spaces. Intracellular water which is calculated from fetal total water minus the extracellular water came to around 46 %. Our observations are on the whole cerebral matter; we are, therefore, far from understanding the water content of any particular kind of cells of the brain.

The electrolyte concentrations of the human fetal brain shows a higher water, ash and sodium content at earlier periods than at later periods of gestation, in fetal brain potassium contents were lower than sodium; whereas in adult brain the contents were around the same. Intracellular fluid of the fetal brain sodium and potassium was found to be present in almost equal concentrations at earlier periods of gestation. In adult cat cerebral cortex [32], intracellular sodium could be calculated to be only a small amount of the intracellular potassium contents.

Glucosamine-6-Phosphate Synthetase

In fetal life, the glucosamine-6-phospahate synthetase activity of the liver was more than double that of adult liver. This enzyme starts the reaction sequence necessary for the synthesis of the AMPS and is the rate limiting step. The high activity in fetal life is consistent with the AMPS content in this period of life. The activity of their enzyme in the liver is many times higher than the activity in several other growing organs. The high quantity of Glm-6-P synthesized. Glucosamine-6-phosphate synthetase activity of the fetal lung was only about a fifth of the activity of the liver. Whereas there was no reduction of the liver enzyme activity with progress of gestation. The AMPS content of the fetal lung. Admittedly, one of the chief functions of the Glm-6-P synthetase in to provide the glucosamine for synthesis of the AMPS. It is also true for adult liver that the Glm-6-P synthesized in the liver undergo dephosphorylation to 9 Glm and the Glm is utilized elsewhere. Glm-6-P synthetase activity of brain and also of the lung was lower than that of the liver. When expressed per gm. of tissue the activity of the liver was between 12 and 22 times that of the brain; when expressed per gm. of protein the hepatic activity was between five and seven times that of the brain. We did not have any adult brain to analyse for the enzyme activity. So we had to be content with determination of the enzyme content in fetal brain upto 24 weeks of gestation.

Conclusions

An organ consists of a supporting framework which is permeated by the characteristic cells of the organ and vascular network. A growing organ of the fetus is at first characterized by condensation of the mesodermal and endo-or ectodermal cells, which divides and differentiates into the tissues and cells of the particular organ. It has been envisaged that, at first, there is proliferation and differentiation of mesenchymal cells, along with the characteristic cells of the organ. The function of the mesenchymal cells is to provide a suitable matrix formed by the mucopolysaccharides, collagen fibers and the fibroblast and mast cells. This complex matrix is permeated three dimensionally by the characteristic cells of the organ. This matrix constituting the interstitial material undergoes variation in composition as the organ grows, divides, differentiates, and becomes mature. The object of the enquiry has been to study some of the properties of this matrix in three human fetal organs, namely the liver, lungs and brain. In all three organs the total water content was higher in fetal life than in adults. Water was present at the highest concentration at the earliest gestation period that could be studied. It gradually decreased with the progress of gestation. The thiocyanate space was found to be much higher in fetal organs than in adult organs; the intracellular water in fetal and adult organs was more or less the same. Thus the higher water content of fetal organs could be entirely accounted by the extracellular compartment. The acidic mucopolysaccharides of the liver was present in almost twice the concentration found in neonatal livers and the extracellular fluid in fetal life was also about twice that in adult liver. Thus there was a positive correlation between the acidic mucopolysaccharides and the

extracellular fluid. Perhaps the water molecules were bound in the interstices of the polyanions as hydronium ions (H_3O^+). The decrease of acidic mucopolysaccharides with progress of gestation was found to be due to a decrease of hyaluronic acid which was more concerned with water binding. The total extracellular sodium and potassium contents in fetal liver was higher than in adult livers and intracellular potassium was lower. The higher extracellular water in fetal livers attracted the hydronium ions and the sodium and potassium ions in order to maintain proper osmotic tension. These cations, in turn, attracted anions in order to preserve electrochemical neutrality. The total osmotic tension in fetal organs was found to be somewhat higher than in adult organs, which finding may have something to do with the buoyant density of the fetus in the amniotic fluid. Total mucopolysaccharides of the fetal lung was much higher than in fetal liver, so also was the total water content. There was a progressive decrease in the water and hyaluronate contents of the lung as gestation advanced. As in the fetal liver, the fetal lung had an increased thiocyonate space, where the acidic mucopolysaccharides attracted hydronium ions and other counter ions like sodium and potassium. A considerable quantity of intracellular sodium was calculated to be present in the lung for which no explanation could be given. Intracellular potassium content was lower in fetal lung than in adult lung. In fetal brain the total acidic mucopolysaccharide content was higher than in fetal liver. There was no measurable decrease of total acidic mucopolysaccharides and hyaluronates from 9 to 28 weeks of gestation and the water content also did not change much during this period. In neonatal and adult life the brain had lower acid mucopolysaccharide, hyaluronate and water contents. In fetal brains the extracellular and intracellular water were found to be equal in amounts. Sodium was present mostly in the extracellular compartment. The intracellular potassium content in fetal brains was lower than reported adult values. During the biosynthesis of mucopolysaccharides, the primary enzymatic reaction is amination of fructose-6-phosphate brought about by the enzyme glucosamine-6-phosphate synthetase. Upto the year 1978, there has been no report on the activity of this enzyme in human fetal organs. An assay system has been developed to measure the activity of this enzyme. Some properties of the enzyme have been studied and its purification has been partially successful. The activity of the enzyme was highest in the fetal liver, about five to seven times that of the fetal lung or brain. The fetal liver had also 2.5 times the activity of the adult liver. It appears, therefore, that the glucosamine-6-phospahte synthesized in the liver undergoes dephorphorylation to glucosamine which is made available to other tissues for the necessary biosynthesis of the mucopolysaccharides and other compounds.

Summary

(A) The acidic mucopolysaccharides, especially the hyaluronate content of human fetal organs bore a positive correlation to the water content; probably the polyanions became associated with the hydromium ions.
(B) The anionic macromolecules formed complexes not only with hydronium ions but with other cations like sodium and potassium which in turn attracted other anions like chlorides, bicarbonates etc.
(C) The human fetal organs had higher osmotic tension in their body fluids than human adult organs, which may have something to do with the buyant density of the fetus in the amniotic fluid.
(D) The glucosamine-6-P synthetase, the primary rate limiting enzyme in the biosynthesis of mucopolysaccharides was present in a very high concentration in the fetal livers.

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Urea Biosynthesis in the Human Fetal Liver

9

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Introduction

There are three types of animals: (1) Ammoniotelic, (2) Uricotelic, and (3) ureotelic. All mammals are Ureotelic.

Ammonia which is one of the main substrates producing urea is toxic to the system. There are a number of ways by which ammonia is produced in the system of mammals.

Kerbs [1] demonstrated that the mammalian liver and kidney catalyzed the oxidative deamination of both D- and L- amino acids. It was shown that L- amino acid oxidases do not have the activity to account for the catabolism of all L- amino acids. Except L- glutamic dehydrogenase, there is very few known L- amino acid oxidative system in mammals. But transamination reaction by transaminases are very active in mammals. On this basis, the mechanism of the L- amino acid oxidation producing ammonia is said to be by involvement by transamination of L- amino acid with α- ketoglutarate producing L- glutamate which is then oxidatively deaminated by glutamate dehydrogenase to form ammonia and α- keto glutarate. The following are the routes by which ammonia can be produced in the mammalian system.

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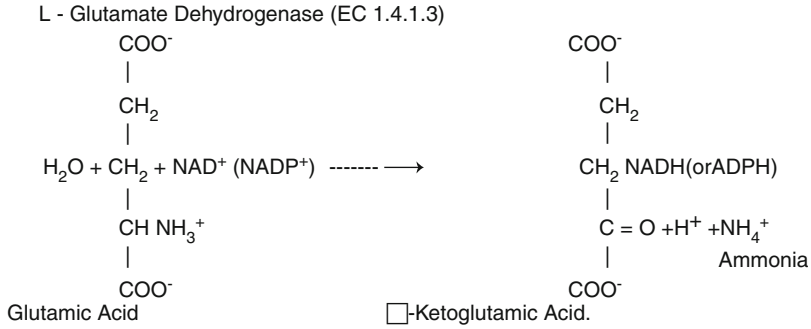


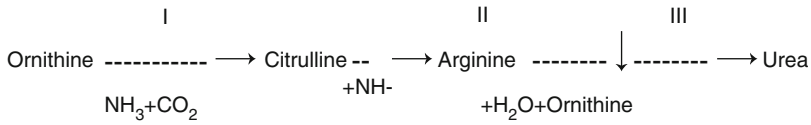
The enzyme glutamate dehydrogenase is a mitochondrial enzyme of widespread distribution. In animal tissues, the activity of the enzyme is high in liver, kidney, brain but low in heart.

That the liver is the site of urea formation in ureotalic organisms was proved by Bollman et al. [2] in 1924.

Sir Hans Krebs and Hanseleit [3], in 1932, demonstrated an entirely novel mechanism of urea synthesis by the tissue slice technique.

Measurement of the rate of the production of urea in the presence of ammonia and other substrates showed that, among a large number of substances tested, two aminoacids, ornithine and citrulline, can under certain conditions, accelerate the rate of formation of urea from ammonia.

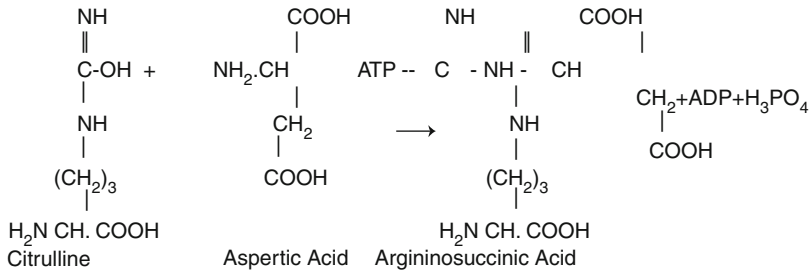
Krebs [4] was led to regard the function of ornithine as that of an intermediate in a series of reactions which would bring about its regeneration and arginine filled a required niche in the concept. On the basis of this hypothesis and the effect of citrulline on liver slices to produce urea from ammonia, it was assumed that citrulline may also be an intermediate in the conversion of ornithine into arginine according to the scheme:



Ammonia and carbon dioxide react with ornithine to form citrulline (Step I). Citrulline then condenses with a second molecule of ammonia to form arginine (Step II). The cycle is completed by the action of arginase (Step III). Under suitable conditions a small amount of ornithine will thus catalyse the formation of a large amount of urea provided the supplies of ammonia and carbon dioxide are adequate.

The reaction of citrulline to arginine could occur in two distinct step – condensation and simultaneous breakdown of the condensation product.

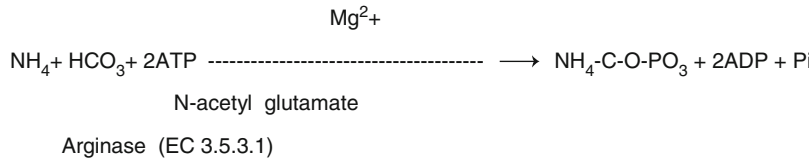
The condensation reaction is said to take place by the condensation of aspartic acid and citrulline in the presence of ATP and Mg^{2+}.



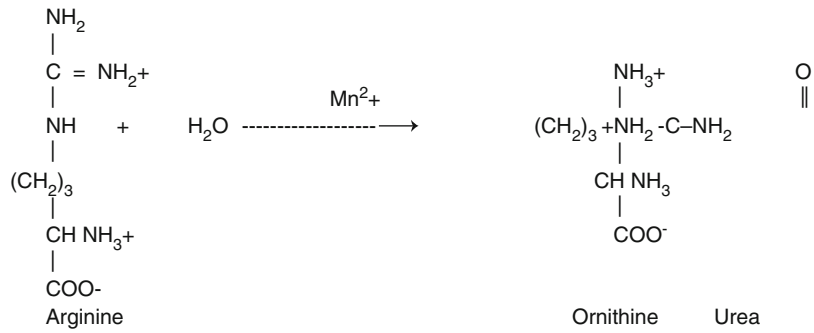
This intermediary condensation product was named as argininosuccinic acid [5]. The enzyme responsible for the formation of arginino succinic acid was named as arginino-succinic acid synthetase.

The enzyme that claves argininosuccinic acid to arginine and fumaric acid is known as argininosuccinase or L-argininosuccinate arginine lyase.

The name of the enzyme proposed for the latter was carbamyl phosphate synthetase. The formation of carbamyl phosphate was then caralyzed by carbamyl phosphate synthetase of liver according to the following reaction [6].



Arginase (L- arginineurohydrolase) is one of the earliest enzyme known. It catalyzes the hydrolysis of arginine to ornithine and urea as shown by the equation.



The enzyme is present in a high concentration in the livers of all ureotelic animals [7].

The location of the enzyme is in the cytoplasm. Co^{2+} and Mn^{2+} are the activators of the enzyme.

Biosynthesis of Urea in Mammals during Development

The evolutionary adaptation of nitrogen excretion was first expressed in an elegant form by Balwin [8, 9]. The nitrogenous excretory products may be varied by metabolic adaptations, with evolutionary branching. Needham [10, 11] suggested that during development, the emoryo excretes ammonia first and eventually develops a mechanism for nitrogen excretion characteristics of the species, recapitulating its evolution. This was supported by Plentin [12] in 1957 who showed that 50-fold decrease in exchangeable water took place in monkeys (Macaque) after birth than in the fetal state. This decrease in exchanging water makes possible the elimination of nitrogen by different methods. Brown and Cohen [13] and Brown et al. [14] showed that during metamorphosis, tadpole changed from ammoniotelism to ureotelism. It would be anticipated that during embryogenesis in mammals, development of the enzymes for urea biosynthesis would take place in preparation for the nonaquatic environment after birth [15].

Among mammals, the enzymes of the urea cycle were extensively studied in rats. Kennan and Cohen [15] and Rahis and Suihkonen [16] showed that the activity of the urea cycle enzymes was very low in fetal rat liver but it showed a rapid postnatal increase. The overall capacity of liver slice to synthesize urea was almost absent from fetal rats and guinea pigs and increased rapidly to adult capacity after birth [17]. Kennan and

Cohen [15] also showed that the enzymes of the urea cycle were present in significant amounts in the liver of the youngest pig embryo studied (28 days). The species difference, which is quite marked in these two groups, viz., rat and pig, was also discussed by Kennan and Cohen [15] in relation to fetal membranes of the two animals and the maturation of the fetus and new born [15]. They (1959) also differentiated the two animals with regard to the maturity of the animals at birth, the development of the mesonephric kidney and the thickness of the placental membrane.

Miller and Chen [18] showed that the activities of the enzymes of the urea cycle are very low in the fetal liver of rats and they increased rapidly after birth; the rate limiting enzyme was again found to be the condensing enzyme of arginine synthetase system, i.e., argininosuccinic acid synthetase. Illnerova and Kubat [19] also investigated whether there were some factors which could be responsible for the sudden increase of the urea cycle enzymes after the 14th day of its gestation and also for the postnatal increase of the enzymes.

Slemons and Morriss [20] studied the maternal and fetal serum concentrations of non-protein nitrogen and urea and showed that there was no difference in concentrations in these two compartments in human beings and rats. The direct measurements of the production of urea was made by Manderscheid [21] and Kennan and Cohen [15] by using the tissue slice techniques of Krebs and Henseleit [3] and they observed that urea could be produced in liver slices from human fetal liver at 3–4 months of gestation. In humans, urea can cross the thin fetal membrane system to the mother efficiently, for there is no allantoic vesicles in the human being [15].

From the proceeding brief review, it appears that although urea biosynthesis in human fetuses has been studied by various observers, there is need for further study in order to evaluate the role of the fetal liver in making urea and also to use this parameter as an index of biochemical differentiation of the fetal liver. Our group of researchers led by Prof. K.L. Mukherjee, studied the human fetal liver at various stages of gestation at SSKM Hospital, Calcutta, from 1977 onwards, as mentioned in Chapter 6. The fetuses were obtained from consenting mothers undergoing hysterotomy under the MTP Act or for other reasons. Due ethical consent was obtained from the then institutional ethical committee. The object of our study was:

1. Measurement of urea and ammonia nitrogen concentrations in the bladder fluid of fetuses.
2. Measurement of the activities of ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinase and of arginase in the livers of these fetuses.
3. Measurement of the concentration of ornithine in fetal livers.
4. Some studies on the metabolism of ornithine in fetal livers.

Ammonia Nitrogen and Urea Nitrogen Concentrations of the Bladder Fluid and Plasma

All the fetuses studied in the present series contained some fluid in the bladder. Amount of the fluid varied from fetus to fetus and cannot be taken as an indication of maturity of the fetus. Although it was generally true that the amount of fluid obtained in fetuses of earlier gestation period was smaller in quantity than in the more matured fetuses, fetuses of similar weights had widely varying amounts of bladder fluid. Ammonia nitrogen and urea nitrogen concentrations of the bladder fluid were determined in 23 fetuses; Results are shown in Table 9.1. The ammonia nitrogen concentrations varied from 0.23 to 1.25 mg per 100 ml. There was no correlation between body weight and ammonia nitrogen concentrations of the bladder fluid. The same thing was observed with urea-nitrogen concentrations of the bladder fluid. It varied from 8.5 to 24.4 mg per 100 ml. Here also, there was no correlation between body weight and urea nitrogen concentrations. Table 9.2 showed that concentrations of ammonia nitrogen and urea nitrogen did not change with gestation.

The ammonia nitrogen concentrations of the plasma of seven human fetuses and their mother's were determined along with the urea nitrogen concentrations. Results are shown in Table 9.2. In this table is also shown the composition of the urine passed on the first day after birth and the

composition of the bladder fluid. It appears from the data that the urea content of the bladder fluid was more or less the same as that of the fetal and maternal plasma. The ammonia nitrogen concentration of the bladder fluid was somewhat higher than that of the fetal plasma which was more or less the same as that of the maternal plasma.

Table 9.1 Concentration of ammonia-nitrogen and urea-nitrogen with respect to different gestational age and corresponding body weights

Gestational age (weeks)	Body weights (grams)	Ammonia-nitrogen	Urea-nitrogen
9–12	6–15(0)	x	x
12–15	15–65(0)	x	x
15–18	65–255(6)	0.42 + 0.064	15.0 ± 1.12
18–21	255–377(4)	0.6 ± 0.064	11.1 ± 1.79
21–24	377–630(5)	0.61 ± 0.165	17.9 ± 2.74
24–27	630–855(4)	0.36 ± 0.054	11.0 ± 0.22
27–30	855–1,490(4)	0.57 + 0.079	16.1 + 2.26

Table 9.2 Composition of bladder fluid, newborn urine, fetal and maternal plasma at 20–28 weeks of gestation

	Bladder fluid	New born urine	Fetal plasma	Maternal plasma
Urea	35	378	29	32
Ammonia	0.42	–	0.045	0.04
Sugar	37	Nil	45	93
Sodium	145	359	127	132
Potassium	4.7	12.7	3.7	4.9
Chloride	99	279	105	93

Enzyme Activity

Ornithine Transcarbamylase during Development

Activities of ornithine transcarbamylase in the livers of the fetuses are shown in Table 9.3. Activities were low in the fetuses of earlier gestation periods. There appear to be no period in the life of the fetus when the increase of this enzyme activity was abrupt and more than at other periods. However, the number of samples examined was rather small and hence no definitive statement can be made. The functional differentiation of the liver with respect to this enzyme, appears to take place throughout gestation. Even when the baby weight is about 1.5 kg. and is certainly viable, the ornithine transcarbamylase activity is less than half of the adult value and the specific activity is only a fifth.

Activities of Argininosuccinic Acid Synthetase during Development

This method of measurement of argininosuccinic acid synthetase activity involves the determination of citrulline concentration before and after incubation. The difference found was never too high.

The activity of this enzyme followed the pattern for ornithine transcarbamylase rather closely. The activity of argininosuccinic acid synthase per gram wet weight of liver was lowest in

Table 9.3 Activities of ornithine transcarbamylase in developing human fetus

Gestational ages (weeks)	Body weights (grams)	Activity Range	Specific activity Range	Sp. activity (fetus) Sp. activity (adult)
9–12	6–15 (3)	85.4–100.2	1.1–1.5	1/32
12–15	15–65(3)	104.0–120.0	1.5–1.8	1/26
15–18	65–255(5)	150.5–278.0	1.7–5.2	1/13
18–21	255–377(6)	329.5–387.0	4.8–5.5	1/8
21–24	377–630(4)	229.0–472.5	3.52–6.47	1/8
24–27	630–855(3)	457.5–480.3	7.05–7.19	1/6
27–30	855–1,490(4)	674.1–749.6	8.2–9.2	1/5
Adult 29 years				1
Adult 55 years				1

N.B.: * Activity is expressed as micromole product per gram wet weight
Specific activity is expressed as micromole product per milligram protein

the smallest fetuses and increased with increasing gestational age (Table 9.4). The activity of the enzyme per gram of wet weight of liver was 13.09 in fetuses of 12–15 weeks of gestation and increased with progress of gestation at the highest gestational age, viz., during 27–30 weeks of gestation to 81.97. The specific activity, i.e., the activity per milligram of protein was also low ranging from 0.165 in the earliest fetuses studied to 1.17 in the 27–30 weeks of gestation. We could get only two livers from adult persons who were 29 and 55 years old. The activity found was 90 and 143 units respectively pre gm of liver. The specific activity however was rather close in the two subjects.

Activities of Argininosuccinase during Development

The method of estimation of argininosuccinase activity involves the determination of arginine by the conversion of latter to urea by added arginase to the system.

The results of determination of argininosuccinase activity in the livers of fetuses of different gestation periods are shown in the Table 9.5. The enzyme activity could not be carried out in the very small fetuses. From 12 to 15 weeks of gestation the enzyme activity steadily increased to 24 weeks. The increase was real in as much as the specific activity was also found to increase. At about 30 weeks of gestation the enzyme activity per gram of wet weight of the liver was almost the same as that found in adults but the specific activity was half that of adults.

Activities of Arginase in Developing Human Fetuses

Both the enzyme activity per gram of fresh tissue as well as the specific activity were more or less the same throughout the gestation periods investigated namely 9–30 weeks, except perhaps in the very small fetuses, where the activity found was somewhat low. These values are shown in Table 9.6. As compared to the adult values, the enzyme activity of the fetal livers was almost half of that in the adult.

The Probable Rate in Which Urea Can Be Produced

Five enzymes are consecutively required for the production of urea from ammonia, the product of one reaction serving as the substrate for the next. We will consider the last four enzymes in this sequence. The rate of ornithine transcarbamylase at 15 weeks of gestation is 1/32nd of the rate in the adult. We, can, therefore, assume that the

Table 9.4 Activities of argininosuccinic acid synthetase in developing human fetuses

Gestational ages (weeks)	Activity range	Specific activity range	Sp. activity (fetus) / Sp. activity (adult)
9–12	Not done	Not done	Not done
12–15	10.56–17.2	0.13–0.22	1/30
15–18	13.4–34.0	0.19–0.495	1/14
18–21	31.7–45.7	0.46–0.64	1/9
21–24	30.1–39.5	0.41–0.58	1/10
24–27	38.0–68.04	0.5–1.036	1/7
27–30	70.4–89.5	0.99–1.4	¼
Adult 29 years	90.0	4.74	1
Adult 55 years	142.8	5.30	1

N.B.: * Activity is expressed as micromole product per gram wet weight
Specific activity is expressed as micromole product per milligram protein

Table 9.5 Activities of argininosuccinase in developing human fetuses

Gestational ages (weeks)	Activity range	Specific activity range	Sp. activity (fetus) / Sp. activity (adult)
9–12	Not done	Not done	Not done
12–15	14.1–20.8	0.2–0.29	1/9
15–18	13.5–32.2	0.29–0.52	1/6
18–21	32.0–49.8	0.52–0.71	¼
21–24	38.4–64.6	0.5–1.01	1/13
24–27	62.2–77.4	0.93–1.03	½
27–30	62.3–88.0	0.69–1.1	½
Adult 29 years	88.3	2.21	1
Adult 55 years	55.0	2.40	1

N.B.: * Activity is expressed as micromole product per gram wet weight
Specific activity is expressed as micromole product per milligram protein

product is formed at 1/32nd of the adult rate. The rate of the next enzyme, the arginosuccinic acid synthetase is also 1/30th of the adult rate. The problem now is whether the product of the first reaction is sufficient to saturate the second enzyme to form ES complex? Since the second enzyme activity was found reduced to also the same extent, the reduction may be assumed to involve either the amount of the enzyme or the presence of an inhibitor. In the former event, the reduced amount of the product of the first reaction may or may not be sufficient to saturate the second enzyme. In the latter event the rate of the overall reaction occurring as a result of the combined action of the first and second enzymes will be found to be successively affected. From many considerations, it appear that the overall rate of the reaction is successively affected in the sequence of reactions.

Since the reactions are mutually dependent the laws of conditional probability was used to calculate the final overall rate of urea production from ornithine and carbamyl phosphate to urea (Table 9.7). To take an example, the probability of a molecule of carbamyl phosphate forming citrulline is 1/32nd of the adult rate; the probability of citrulline forming argininosuccinic acid is 1/30th of the adult rate. Eventually, it can be argued that the probability of a molecule of carbamyl phosphate going on to urea is 1/14,598 of the adult rate in a fetus of 12–15 weeks to 1/104th of the adult rate in a fetus of 27–30 weeks.

Table 9.6 Activities of arginase in developing human fetuses

Gestational ages (weeks)	Activity × 10^3 range	Specific activity range	Sp. activity (fetus) / Sp. activity (adult)
9–12	6.93–10.02	40.8–70.6	Not done
12–15	8.734–18.76	67-13-120.0	1/3
15–18	11.5–16.8	80.8–98.8	½
18–21	10.32–19.7	86.0–126.0	½
21–24	12.337–18.816	92.2–120.0	½
24–27	12.264–25.872	91.5–134.0	½
27–30	11.2–25.5	85.0–118.0	1/2
Adult 29 years	48.72	193.3	1
Adult 55 years	50.75	193.5	1

N.B.: * Activity is expressed as micromole product per gram. Wet weight
Specific activity is expressed as micromole product per milligram protein

Concentration of Ornithine in Human Fetal Liver

Since arginase activity of the fetal livers was comparatively higher that the other enzymes of the urea cycle and since arginine was presumably available to the fetus through the placental circulation, it appeared reasonable to study the metabolism of the products of arginase activity. The urea being a highly diffusible substance was probably eliminated by the maternal kidneys. The level of free ornithine in the liver was, therefore, studied in fetuses of different gestation periods.

Table 9.7 Probability of the rate of urea production in human fetuses

Gestational age (weeks)	Ratio of specific activities (fetus/adult)				Probability of the rate of urea production fetus/adult
	Ornithine transcarbamylase	Argininosuccinic acid synthetase	Argininosuccinase	Arginase	
9–12	1/32	X	X	1/3	X
12–15	1/26	1/30	1/9	½	1/14,598
15–18	1/13	1/14	1/6	½	½,364
18–21	1/8	1/9	¼	½	1/656
21–24	1/8	1/10	1/3	½	1/552
24–27	1/61	1/7	½	½	1/200
27–30	1/5	1/4	1/2	1/2	1/104

We estimated the ornithine content by employing a partially purified ornithine transcarbamylase and estimating the citrulline produced.

The concentrations of free ornithine in human fetal livers are shown in Table 9.8. It varied from 0.22 to 0.58 μmol/g of wet weight of liver. There was no correlation between the concentration of ornithine and gestational age. In most of the cases, the concentration of ornithine was found to vary from 0.2 to 0.3 μmol/g wet weight of liver. It appears from these data that the concentration of ornithine remained almost constant throughout the gestation of ornithine in the adult. The concentration of ornithine in fetal livers was between two and four times than that of reported values for adult livers [22].

Since the free ornithine content of fetal livers was found higher than the reported values of adult livers, it was thought worthwhile to enquire into the contents of polyamines in fetal livers since they are synthesized from ornithine.

Table 9.8 Content of ornithinine in human fetal liver

Sl. No.	Body weight (grams)	Gestation period (weeks)	Micromole of ornithine/gram wet weight of liver
1.	175	17	0.28
2.	295	20	0.22
3.	330	21	0.52
4.	420	22	0.27
5.	590	24	0.58
6.	660	25	0.246
7.	1,010	28	0.2
8.	1,450	30	0.32

Concentration of Polyamines in Human Fetal Liver

The polyamines were extracted from the livers and separated by high voltage electrophoresis. Results are shown in Table 9.9.

The concentrations of putrescine and spermine expressed in micromoles and millimicromoles per gram of wet weight of liver in the Table. Concentration of putrescine increased from 0.34 μmol in the fetuses of 15–18 weeks of gestation to 3.08 μmol in the gestation period of 21–24 weeks. The concentration of this polyamine decreased to 1.2 μmol per gram of wet weight of liver in the higher gestation periods. The increase of putrescine concentration from 12 to 24 weeks and its subsequent decrease could not be fully ascribed to the higher water content of the liver in the fetuses of earlier gestation periods. When the concentrations were expressed per 100 g of dry weight, then also the same increase and the subsequent decrease was demonstrable.

The concentration of spermine on the other hand steadily increased from the earlier gestation period to the late gestation periods. The concentration of spermine was expressed in millimicromoles per gram of wet weight of liver were as those of putrescine were expressed in micromoles, indicating the higher concentration of the latter polyamine in fetal livers. Spermine content increased from 9 millimicromoles per g to 113.7 millimicromoles per g with the increase of gestational age from 15 to 30 weeks. The steady increase in the spermine with increase of gesta-

Table 9.9 Concentration of polyamines in human fetal liver

Gestational ages (weeks)	Putrescine		Spermine		Molar ratio	
	Micromoles per gram. Wet weight	Micromomes per 100 g dry weight	Millimicromoles per gram. Wet weight	Micromoles per 100 g. Dry weight	Putrescine wet weight/ spermine dry weight	
9–12	X	X	X	X	X	X
12–15	X	X	X	X	X	X
15–18 (5)	0.34 ± 0.11	200	9 ± 0.81	5.3	37.7	37.7
18–21 (4)	1.84 ± 0.8	920	50.01 ± 5.1	25.0	36.7	36.8
21–24 (6)	3.08 ± 0.6	1,400	66.6 ± 12.2	30.0	46.2	46.6
24–27 (6)	1.127 ± 0.73	427	85.91 ± 47.5	35.7	13.1	11.9
27–30 (7)	1.2 ± 0.19	500	113.7 ± 51.0	47.1	10.5	10.6
3 years	1.217	420	103.0	35.5	11.8	11.8

tion was also apparent even when the data were expressed per 100 g of dry weight. The ratio of putrescine to spermine which was high at first, gradually came down to a steady proportion from 24 weeks of gestation onward.

In Table 9.10, the concentrations of putrescine and spermine were expressed per milligram of DNA in the human fetal liver and the results were compared with the concentrations of RNA per milligram of DNA in the same fetuses. Even then, the results are reminiscent of the earlier mode of expression, i.e., per milligram of tissue weight. The putrescine level increased from 0.009 µmol at 15 weeks to 0.37 µmol at 30 weeks. In the 3 year old child, the level was 0.32 µmol.

Spermine concentrations per milligram DNA steadily increased from 0.25 µmol at 15 weeks to 11.7 millimicromoles at 30 weeks. In the 3 year old child the level was 27.5 millimicromole.

RNA concentrations per milligram of DNA was more or less the same from 15 to 30 weeks of gestation. The concentration was higher in the liver of the 3 year old child.

Activities of Ornithine Amino Transferse (EC.2.6.1.13) during Development of Human Fetuses

The activity of this enzyme was studied in a small number of fetuses, upto 24 weeks of gestation by the method described by Jenkins and Tsai [23]. No activity was found in any of the fetuses studied.

Discussion

In the present investigation, the composition of the bladder fluid shows that urea concentration of the bladder fluid was more or less the same as that of the fetal and maternal plasma. The fluid that is filtered at the glomerulus naturally contains an ultrafiltrate of plasma and, therefore, the glomerular fluid contains urea in the same concentration as it is present in the fetal plasma. The fluid is reabsorbed in the proximal tubules in an iso-osmotic concentration so that when the fluid reaches the descending limb of the loop of Henle the osmolar concentration is the same as that of the glomerular filtrate as it formed. The urea concentration is also the same as that of the plasma. Later on, when the water gets reabsorbed in the loop and the distal tubules the urea concentration of the urine also increases proportionally. Since in the fetal bladder fluids, urea concentration was the same as that of the fetal plasma it can be presumed that there was no reabsorption of water in the distal tubules. Whether there was reabsorption in the proximal tubules or not it is difficult to say, because in either case the osmolar and urea concentrations of the fluid will be the same as that of the ultrafiltrate.

Table 9.10 Comparison of concentrations between polyamines and ribonucleic acid per milligram of deoxyrihonucleic acid during development of human fetal liver

Gestational ages (weeks)	Putrescine	Spermine	Molar ratio putrescine/spermine	Ribonucleic acid
9–12	X	X	X	X
12–15	X	X	X	X
15–18 (5)	0.0094 ± 0.004	0.25 ± 0.1	37.6	0.369 ± 0.107
18–21 (4)	0.1505 ± 0.06	4.56 ± 1.23	33.0	0.321 ± 0.073
21–24 (6)	0.3705 ± 0.01	6.88 ± 1.75	53.8	0.349 ± 0.082
24–27 (6)	0.128 ± 0.088	9.16 ± 4.8	14.0	0.335 ± 0.071
27–30 (7)	0.16 ± 0.085	11.7 ± 4.7	13.7	0.385 ± 0.088
3 years	0.3245	2,746	11.8	0.54

Results are expressed as means ± S.E.M. to the number of fetuses indicated in parenthesis. Concentrations of putrescine and spermine are expressed in micromoles and millimicromoles respectively per milligram of DNA. RNA is expressed in milligram per milligram of DNA

The ammonia concentrations of the bladder fluid, although higher than that of the fetal plasma, was nowhere around the concentrations found in new born or adult urine. Urine is a freely diffusible substance; in fact it has been used to determine the total body water concentration. The exchange of urea across the placenta is, therefore, complete. The fetal plasma, in fact contains urea in the same concentration as that of the maternal plasma. If any urea is added to the fetal circulation in the course of fetal metabolism it would eventually find its way to the maternal circulation and be dealt with by the mother. The urea concentration of the fetal plasma, would not, therefore, increase as a result of addition by the fetus and cannot be used as critical of urea production by the fetus, even though fetus has no direct access to the environment to discharge its urinary contents. The fetal bladder fluid closely corresponds to an ultrafiltrate of fetal plasma in as much as it contains more or less the same concentrations of sugar, sodium, potassium and chloride. Sabrazes and Fauquet [24] showed that urines formed in utero had urea but the concentration found was extremely low and less than 50 mg per 100 ml. Barlow and McCance [25] also showed that fetal urines contained total non-protein nitrogen to the extent of 41–61 mg per 100 ml.

The total amount of the fluid in the fetal bladder probably increased with gestation but the concentration of urea nitrogen and ammonia nitrogen remained constant throughout the gestation. Rubatelli and Formentin [26] as already described showed that the concentrations of various non-protein nitrogeneous compounds in the umbilical vein and artery and antecubital vein of the human fetus were similar to that of a normal person. Urea nitrogen and ammonia nitrogen excreted in the urine per day are 10–15 g and 400–1,000 mg respectively. A normal adult excretes an average of 1,500 ml of urine per day. So if the concentrations be expressed in 100 ml of urine, the concentrations of urea nitrogen and ammonia nitrogen are 700–1,000 mg and 26–67 mg respectively. The concentrations of urea and ammonia nitrogen found in the bladder fluids of these fetuses were far from the concentrations found in the urines elaborated after birth. The fluid in the urinary bladder of such fetuses can, therefore, not be properly be called urine, although the ultrafiltration must have occurred through the glomerular filter. No selective reabsorption in the tubule had taken place. It is of some comparative interest to note that in the marine teleost fish which do not synthesize urea, the functional adult kidney has been lost. Birds and saurian reptiles, which are uricotelic, have a marked reduction in the number of glomeruli and of course, lack of a functioning urea cycle.

Thus there would appear to be some relationship between the ability to synthesize urea and the degree of kidney development. Kennan and Cohen [15] thought that the placental mammals apparently began to synthesize urea when the embryonic glomerual kidney had developed and the time of the appearance of urea cycle enzymes might in turn be correlated with the thickness of the fetal membrane system, the presence and size of the allantoic vesicle and the relative maturity of the new born animal.

For the conversion of urea from ammonia, five enzymes, viz., carbamyl phosphate synthetase, ornithine transcarbamylase, argininosuccinic acid synthetase, argininosuccinase and arginase are required in sequence. The product of one enzyme serves as the substrate for the next. Of these five enzymes, the first enzyme carbamyl phosphate synthetase was not measured in this study. Raiha and Suihkonen [27] measured the activity of this enzyme and found it to increase with increasing gestational age, thus producing carbamyl phosphate. There are two major reactions in which carbamyl phosphate participates, namely pyrimidine biosynthesis and urea formation. The synthesis of carbamyl phosphate brings the product to a branch point and hence is subjected to autoregulation.

It is not surprising to find that there seems to be a reciprocal relationship between the activities of the two enzymes, aspartate and ornithine transcarbamylase, both utilizing carbamyl phosphate as their substrates. The former one is necessary to produce pyramidine precursors and the latter enzyme produces citrulline in the formation

of urea. During regeneration of the liver the activity of aspartate transcarbamylase increased while that of ornithine transcarbamylase decreased [28]. Charbonneau et al. [29] also found that during embryonic life in the rat, the activities of all the enzymes of the urea cycle were very low, but aspartate transcarbamylase activity was relatively high. It was claimed that the mother synthesized and eliminated urea, sparing the embryo of these functions. The embryo would, however, have to synthesize nucleic acids for growth and hence pyramiding biosynthesis from carbamyl phosphate would be obligatory. Kretchmer et al. [30] showed that the *de novo* pathway for the synthesis of pyrimidines was at least as active in the fetal liver of the developing animals like rat, rabbit etc. as it was in the adult and that there was no relation between this pathway and that of urea biosynthesis in liver. Hager and Jones [31] suggested from their experiment that the glutamine dependent carbamyl phosphate synthetase provided carbamyl phosphate for the pyrimidine pathway. Rapidly growing normal tissues (fetal liver, spleen, testis) have much more carbamyl phosphate synthetase II activity that other tissues (lung, uterus, brain, heart and muscle) [32]. In the regenerating liver and also in fetal rat liver ornithine transcarbamylase was found to be low [28]. When the regeneration was complete or after the rat was born, the aspartate transcarbamylase activity relatively decreased and the activity of ornithine transcarbamylase correspondingly increased thus channeling the carbamyl phosphate along one or the other route according to the metabolic requirement characteristic of the particular condition. The urea cycle starts from the carbamyl phosphate synthetase I activity: actually speaking the characteristic reaction starts from ornithine transcarbamylase. As is so often the case in a chain reaction like in the biosynthesis of urea, the first step is usually the rate limiting reaction. In this cycle, therefore, the ornithine transcarbamylase may be considered to play a rate limiting role in the sequence of reactions ultimately ending in biosynthesis of urea.

The activity of ornithine transcarbamylase in human fetal livers was found to be very low in fetuses of early gestation period. It is very easy to find a teleological explanation for this phenomenon. Since the fetus is a rapidly growing organism it needs to synthesize the nucleic acid pyrimidines and, therefore, all the carbamyl phosphate is channelized to pyrimidine biosynthesis.

The growth of the human fetal liver mass with respect to time is more or less uniform [33]. For example the liver represents 44 mg per g of body weight at 12 weeks and 38 mg per g at 32 weeks. The slight decrease that occurs may be accounted for by a relative decrease of water. Even when the baby is 6 months old it still weighs 46 mg per g of body weight. Even though the mass of the liver increases proportionately to the mass of the body weight, there is a definite decrease of relative amounts of DNA in the liver [34]. For example, the DNA represents 0.87 g % in the fetuses at 14 weeks and 0.50 g % in the fetuses at 28 weeks. Thus with continuing growth of body weight, there is relative decrease of DNA and hence the number of cells per unit mass of the liver. The cells, therefore, accumulate matter probably at the expense of water.

One of the ways of accumulation of matter will be to increase the protein content and hence presumably of the enzyme amount. To take the instance of ornithine transcarbamylase during the growth of the human fetus, can we assume that the relatively undifferentiated liver cell of early gestation period produces little enzyme whereas the amount of the enzyme increases with differentiation. The DNA of the liver cell containing the operator gene of ornithine transcarbamylase in the early period of gestation lies unmasked only in 3 % of the liver parenchymal cells so that they can be transcribed and translated; in the other 97 %, the gene is blocked. The process of differentiation, therefore, can be interpreted as unmasking of the gene for a particular enzyme. However, even at this level we cannot get away from speaking about vague mystery things, of whose nature we do not know. The liver performs its functions remarkably well in fetuses very early in life. For example, it produces plasma proteins of the fetus and synthesizes all kinds of intermediates of biosynthesis for the overall economy of the fetus even at 12 weeks of gesta-

tion. Why is it that such a liver performs plasma protein synthesis so effectively but synthesizes ornithine transcarbamylase very poorly? Is there a hierarchy of functions that are to be eventually performed by the liver. We still do not know answers to these questions.

The next enzyme in the sequence, the argininosuccinic acid synthetase behaves in the same way as ornithine transcarbamylase. If we represent the activity found in adults as one, the respective relative activity of ornithine transcarbamylase and argininosuccinate synthetase bears a very close parallelism at each period of gestation. It is possible, therefore, that the operator gene for the two enzymes may be either closely linked or the same in human fetuses. An alternative explanation would be, that unmasking of the DNA for the synthesis of mRNA for both the enzymes occurs by the same substance which may be either an initiator or a derepressor of an inhibitor.

The argininosuccinate synthetase activity is normally about a hundredth of the ornithine transcarbamylase. Hence it is presumed synthetase is the rate limiting enzyme in the urea cycle. In the present experiments both ASA synthetase and ASA-ase activities are about a tenth of the ornithine transcarbamylase; so it is possible that the overall reaction of arginine synthesis will be about a hundredth of the rate of the ornithine transcarbamylase.

The next enzyme in the sequence in urea biosynthesis is argininosuccinase which splits argininosuccinic acid into arginine and fumaric acid.

Relatively speaking, the activity of this enzyme as compared to the activity in the adult was not as low as the other enzymes of the urea cycle except arginse.

When an organ like the human fetal liver functionally differentiates, can we justifiably say that such a functional differentiation occurs in almost all the cells or in a small number of cells comprising a tissue? That there is a specific pattern in the rate of increase of each of these individual enzymes might be interpreted to mean that a certain percentage of cells in assuming the function and the rate of increase is a function of the individual enzyme concerned. Thus the rate of increase of argininosuccinase is different from the rate of increase of the other enzymes of the urea cycle.

The next enzyme in sequence in urea biosynthesis is arginase, which splits arginine into urea and ornithine. Among the enzymes of the urea cycle, the activity of this enzyme was found to be relatively higher than any other enzyme of the urea cycle, when the activity in the fetus was compared to that in an adult. Furthermore, the activity was relatively constant throughout the gestation period studied. That the activity was found to be relatively high even at an early period of gestation could be interpreted to mean that the body uses the products of arginase activity, i.e., ornithine or urea.

The activities of these enzymes in the liver of adult human beings or in the livers of fetuses are more or less in the ranges described by previous workers like Cohen and Brown [35]. It is hard to explain the low value in the fetuses of the different gestation periods.

If the probability of the rate of production of urea by the fetus be compared to that of an adult, it appears that the fetus can produce urea no doubt, but the rate as compared to the adult is negligible. Thus the little amount of urea that is formed probably goes to the mother's circulation to be eliminated in her urine. It is probable that the fetal kidneys do not perform the excretion of urea, as the bladder fluid did not contain any extra urea in any of these fetuses.

Ornithine occurs at a point of intersection of a number of metabolic pathways. It is one of the products of arginase activity on arginine. It can be decarboxylated to yield putrescine. By transamination it can form γ-glitamic semialdehyde. After decarboxylation to putrescine it can form the oligoamines like spermiding and spermine. From glutamic semialdehyde, proline can be formed by reduction.

The discovery that polyamines stimulate growth of microorganisms, and in animal and plant tissues, is of particular interest, and the interaction between polyamines and nucleic acids already studied in cell free extracts probably provides the mechanism for this stimulation [36].

It appears from the Table 9.9 that the concentration of putrescine increases upto a certain gestation period and then decreases whereas the

concentration of spermine increases with the gestational age of the fetus. The sudden drop of putrescine content is also evident from the molar ration of putrescine to spermine, which also increases upto a certain gestation and then falls down. During early fetal life putrescine may be the main requirement because it regulates the synthesis of other polyamines.

In human fetuses the polyamine content is not high in liver cells and therefore cannot be said to perform a stabilizing function on DNA, nor can it have much effect of RNA synthesis either. The control of polyamine synthesis in human fetal livers is unlike that in embryonic chick, toad, rad or sea urchin, where polyamine biosynthesis is definitely increased in embryonic life.

Summary

1. Normal human fetuses of different gestation periods were collected on ice after hysterotomy and the different enzymes of the urea cycle were estimated in the liver. Mother's blood, fetal blood, amniotic fluid and bladder fluids were collected and ammonia nitrogen and urea nitrogen concentrations were measured in these fluids.
2. The urea concentrations of the bladder fluids of these fetuses were very low as compared to the urea concentrations of the adult urines. The values were more or less similar to the values found in the maternal blood and fetal blood.
3. Thus the fluid in the urinary bladder of such fetuses could not properly be called urine although the ultrafiltration must have occurred through the glomerular filter. No selective reabsorption in the tubule had taken place.
4. Of the four enzymes of the urea cycle that were studied, ornithine transcarbamylase had the lowest activity along with argininosuccinic acid synthetase. Their activities increased with progression of gestation.
5. Activity of argininosuccinase was higher than the previous two enzymes, when they were compared to the values found in the adult. It increased with gestation and attained a half of the adult activity at later periods of gestation.
6. Activity of arginase, was found to be the highest in the cycle. The activity of this enzyme in contrast with other enzymes of the urea cycle did not progressively increase with progression of gestation.
7. In the series of reactions ultimately producing urea, since the activity of all these enzymes were found reduced, it appeared that in vivo very little urea was synthesized by the fetal liver, especially in the earlier periods of gestation; the rate increased with progress of gestation but in comparison to the situation in an adult, very little urea was synthesized even in the later periods of gestation. Some urea may be produced by the comparatively higher arginase activity from agrinine, made available to the fetus through the maternal circulations.
8. The very low ornithine transcarbamylase and argininosuccinic acid synthetase activities and comparatively higher arginase activity of the fetal liver might give rise to increased ornithine concentration in fetal liver. The ornithine concentrations were in fact found to be two to four times higher in the fetal liver than in adult livers. Since ornithine does not occur in any body proteins, the ornithine must be metabolized through some other alternate metabolic pathway.
9. Of the two possibilities of utilization of ornithine besides the urea cycle, the proline biosynthesis and polyamine biosynthesis, the former pathway was found to be absent in fetal livers.
10. Some polyamine concentrations were measured in these livers and were found to increase from a very low value at earlier periods of gestation to about a half to a third of the value found after birth. The concentration of putrescine was found to increase upto a certain gestational age of the fetus and then it decreased. Perhaps, the ornithine had to accumulate to some critical value before it could induce the ornithine decarboxylase and putrescine also had to rise to a respectable concentrations before it could stimulate spremidine and spermine biosynthesis.

11. When the concentrations of polyamines were compared with RNA content by expressing the values per milligram of DNA, it was found that RNA concentration per milligram of DNA was more or less the same but the spermine concentration increased with progression of gestation. The ration of putrescine to spermine at first increased and then decreased indicating the formation of spermine in later periods of gestation.

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Mechanism of Rejection of Human Fetal Adrenal Cortex

10

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Introduction

In the course of development from early recognizable fetal stage to about 2 years after birth, the adrenal gland in man and some other primates undergo an interesting sequence of changes, which has been described as physiological involution. The so called involution occurs in what is known as the fetal type of cortical cells. The recognition of fetal adrenal cortex as distinct from the adult cortex is ascribed to Elliot and Armour in 1911 [1]. They found that the adrenal cortex was composed of a narrow rim of cells which constituted the periphery of the cortex and from which the adult cortex was derived. This peripheral zone was named the "Permanent Zone" and the area between the permanent zone and the medulla was occupied by cells which were called the so called "Fetal Cortical Cells". This fetal zone degenerated after birth and was subsequently replaced by regeneration from the peripheral rim of cells.

The human adrenal shows some interesting variations in size and weight at different ages of life. In the fetus for example, it increases steadily in size and is very large in proportion to the size of the fetus. This remarkably large size of the fetal adrenal gland is mainly due to the presence of a well developed fetal zone which involutes after birth.

The organ, therefore, in fetal life is about 20 times heavier than in the adult. At midterm the fetal zone constitutes 85 % of the total adrenal cortex. At birth it is 84 % and 82 % for the still

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born and live born infants, respectively. Two weeks later, the fetal zone is 58 %; 3–12 months later it is only 13 % and seen as a narrow rim [2]. Swinyard [3] and Tahka [4] also found that the permanent zone about the time of birth, occupied 15–20 % of the whole adrenal cortex.

Immediately following birth, there is a spectacular decline in weight; an abrupt 50 % reduction in their mass occurs. The weight of adrenals decreasing after birth appear to be due to the fact that the fetal cortex undergoes a process of degeneration and is not completely replaced until puberty. At the end of the first year, in the human adrenal gland, the fetal cortex has completely disappeared and has been replaced by typical normal cortex but the gland still continues to grow until the individual reaches the age of 20 or 21. Since such a great part of the fetal adrenal cortex persists only during the fetal period, it seems reasonable to assume that the growth and differentiation of this fetal zone are related to some specific functions during pregnancy.

The histological structure of the human fetal adrenal gland differs from that of the adult adrenal gland. The fetal adrenal gland is composed of an outer or true cortical part consisting of a narrow zone of small deeply staining cells arranged in ovoid or acinar groups and an inner portion made up of large polyhedral cells which are frequently fat containing, take a pale acidophilic stain and have no definite arrangement. In the most central region, a few deeply staining medullary cells may be seen.

Keene and Hewer [5] confirmed the derivation of the adrenal cortical cells from the coelomic epithelium, but showed that the fetal and adult type cortical cells arose separately. In the 10 mm embryo, the cells forming the fetal adrenal cortex were already migrating into the mesenchyme, whereas those that were to form the adult type cortex first appeared in the 12 mm stage as a cap overlying the previously differentiated fetal cortex; the extension of this cap to envelop the fetal cortex followed, simultaneously with invasion of the gland with chromaffin cells. Uotila [6] also studied the development of these zones in man and found that fetal cortical cells were first observed in an 8 mm embryo (about 21 days old), whereas the first definite adult-type cortex was observed at 14 mm (about six and half weeks). The primary inner zone of fetal cortex, is present as a definite structure by the fourth fetal month [7] and continues with no morphological change up to the time of birth. Hyperemia is a feature of the fetal cortex at all times and is maximal at the time of birth [8]. The true cortex is relatively richer in nuclei, poorer in cytoplasm and less vascular. The cells are arranged in arcades beneath the fibrous capsule of the gland and resemble those of the adult glomerular zone. These arcades are continuous centrally with columns of cells which are similar to those of the adult fascicular zone, but much shorter. Stoner et al. [9] claimed that differentiation of the adult cortex into zona glomerulosa and fasciculata first appeared between the second and fourth week of fetal life. The adult type reticular zone is absent at birth according to most observers, but develops postnatally [10].

The fetal cortex undergoes a number of changes at birth. The fetal zone degenerates, while the adult zone proliferates and starts to differentiate into three adult zones.

We are proposing a mechanism for the involution of the fetal adrenal cortex. The fetal cortical cells may have surface receptors for placental progesterone or estrogens. The hormones need to be continually replaced. As long as the hormones are bound to the cell surface, the T cells cannot recognize that they are foreign cells; the recognition site is masked. After the placental hormones are withdrawn the recognition site is unmasked. The T cells now recognize them as foreign cells and produce cytotoxic factors which eventually lead to the death of the fetal cortical cells.

We studied the mode of involution of human fetal adrenal cortex in post-uterine life. From the preceding brief review, it appears that the process is due to either a withdrawal of the placental hormones or appearance of a rejection phenomenon of an auto-immune nature. It is proposed in this investigation to study the latter aspect, namely, the non-recognition of a fetal autograft in postnatal life. Therefore, the present investigations are designed to study whether the involution of the fetal adrenal cortex is similar to a graft rejection phenomenon or not. This work was conducted at

the Institute of Post Graduate Medical Education and Research, Calcutta, from 1977 onwards.

The study includes the following aspects-

1. Histological observations on the adrenal glands of human fetuses of 7–36 weeks of gestation and on that of infants and adults.
2. The developmental pattern of soluble proteins of adrenal glands in human fetuses.
3. The possibility of detecting humoral antibodies in the serum of infants against extracts of fetal adrenal cortex.
4. The nature of antigen and antibody reaction in the rejection phenomenon.

Collections and Samples – Normal human fetuses, obtained by hystecotomy, after obtaining due consent as per the norms of the institutional Ethical Committee of the time, were received directly on ice. The adrenals were taken out by dissecting the abdomen of the fetuses. Adrenals were also dissected from children and adults who died of infectious diseases in which adrenals were not affected, and also of accident victims. Blood was collected from the fetuses, infants aged between 6 months to 1 year and adults. Rabbits were used for immunization.

Histological Study

Although the principal object was to study the involution of fetal cortical cells, we studied the development of the adrenal gland as a whole, during the development of the fetus as a part of our study. At a very early stage of development, at about 3 weeks of gestation the presumptive fetal adrenal cortex first appears as a condensation of celomic epithelium at the root of the dorsal mesentery. The islands rapidly proliferate; however all the cells look alike and there is no evidence of any differentiation at this stage. Thereafter, according to Keene and Hewer [5] and Uotila [6], there appear a rim of presumptive permanent fetal zone, which develops separately and then forms a cap around the fetal zone to form ultimately the definitive fetal cortex together. According to others like Gruenwald [11] and Velican [12], the same primitive fetal cortex differentiates into the adult and fetal zones. Bachmann [13] and Lanman [14] have reviewed these morphological aspects.

At About 8 Weeks of Gestation

Figures 10.1 and 10.2 show the histological studies of adrenal gland of the fetus weighed at 1.5 g and was about 8 weeks gestation; it was found that there was a layer of presumptive capsule forming cells, 1 or 2 cells thick, followed by a layer of presumptive adult cortex, 7–8 cells thick, followed by the fetal type of cells 40–45 cells

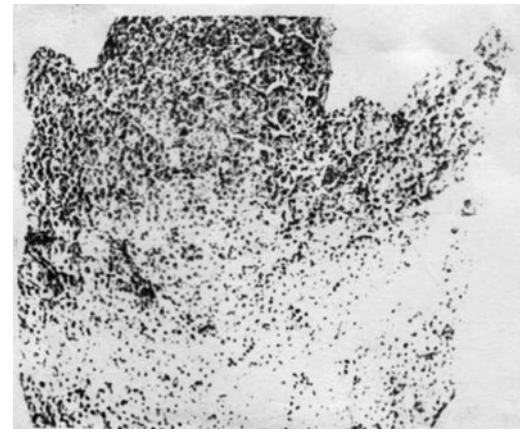

Fig. 10.1 Histological picture of the adrenal gland of a fetus of 8 weeks: the capsular layer, the presumptive adult layer and the fetal layer × 100

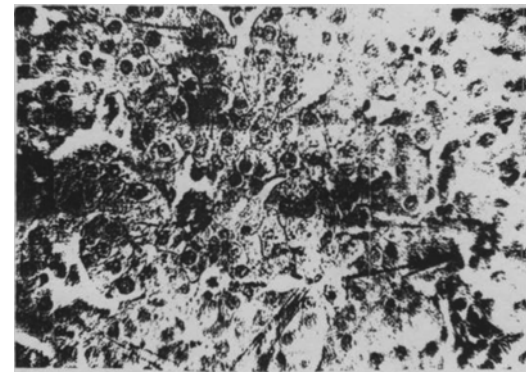

Fig. 10.2 Histological picture of the adrenal gland of a fetus of 8 weeks showing the fetal layer and the islands of blood forming tissue × 400

thick, followed by the 7–8 cells thick adultogenic, and 1–2 cells thick capsulogenic cells. At a number of places in the fetal cortical layer as well as in the adult cortical layer there were islands of blood forming cells without any attempt at capillary formation. The adultogenic layer consists of cells with small and large nuclei and rather smaller cytoplasm as compared to the fetal layer. The fetal layer consists of cells which are larger than the adultogenic layer; the cytoplasm is more in amount and stains more intensely with eosin than the cells of the adultogenic layer. The blood forming cells stain the most with eosin.

At About 9 Weeks of Gestation

The histology of the adrenal gland of a fetus weighing 6.5 g and of about 9 weeks of gestation is shown in Figs. 10.3 and 10.4. From within inwards and then outwards the following layers can be distinguished, i.e., the capsulogenic layer is two to three cells thick and are is more elongated; at places the capsular fibrus connective tissue has been laid down. The adultogenic layer has increased in thickness, i.e., 13–14 cells thick and the fetal layer has also increased in thickness. The blood islands are more in number; some are not enclosed in capillaries. A small number of cells in the blood islands are mature erythrocytes, i.e., non-nucleated cells in contrast to that of the previous fetus (Fig. 10.1) where all the cells were nucleated.

At About 12 Weeks of Gestation

The histology of the adrenal gland of a fetus weighing 14.5 g, at about 12 weeks of gestation is shown in Figs. 10.5 and 10.6. The haematoxylin-eosin (HE) staining shows that the capsule is now well formed. The adultogenic layer consist of cells which are smaller than the fetal cortical cells; the adultogenic layer is very much less vascularized than the fetal layer, which is well vascularized; the fetal layer of cells contains blood in between two rows of cells; the blood contains non-nucleated erythrocytes. Occasionally, within the gland, a very large cell is seen, which for want of terms, I call a "Titan Cell". Its length and diameter are at least three times that of the fetal cortical cells, shown in Fig. 10.7. The capsule contains amylase resistant PAS positive material.

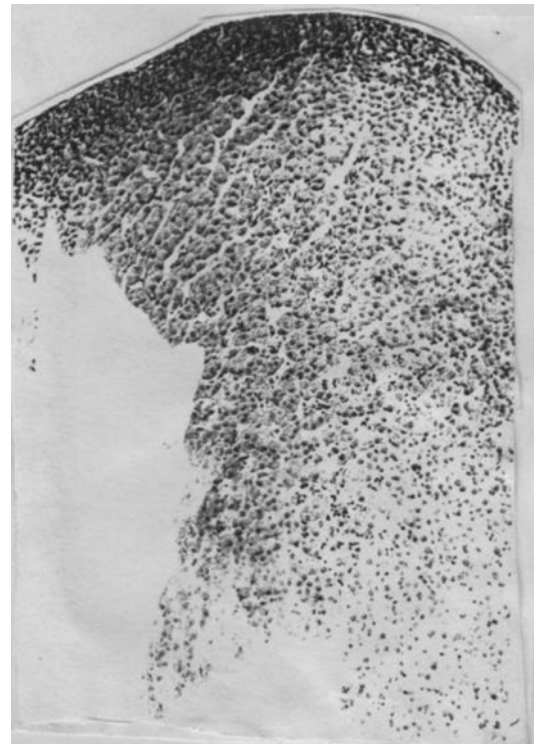

Fig. 10.3 Histological picture of the adrenal gland of a fetus weighing 6.5 g and of 9 weeks: two layers of capsular layer, a small adultogenic zone and a large fetal zone; small islands of blood forming cells are seen, × 100

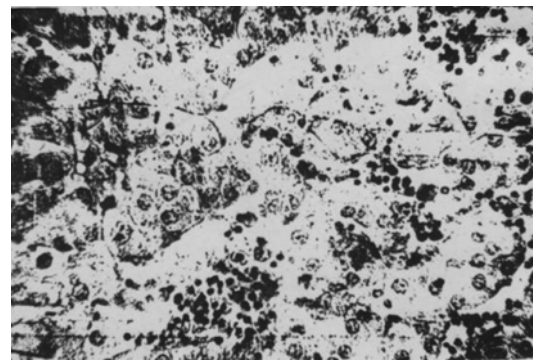

Fig. 10.4 Histological picture of the adrenal gland of a fetus weighing 6.5 g and of 9 weeks of gestation showing fetal zone containg a blood forming island which has few mature erythrocytes, a few erythroblasts and a large number of myeloid cells; × 400

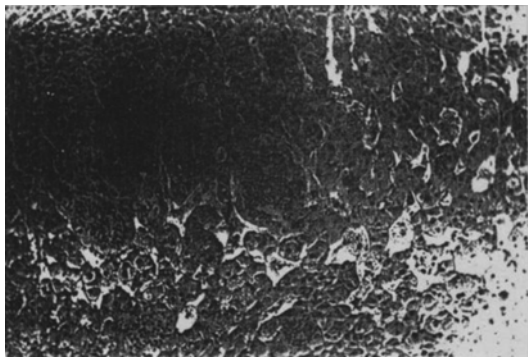

Fig. 10.5 Histological picture of the adrenal gland of a fetus weighing 14.5 g; note the large number of blood forming islands, × 100

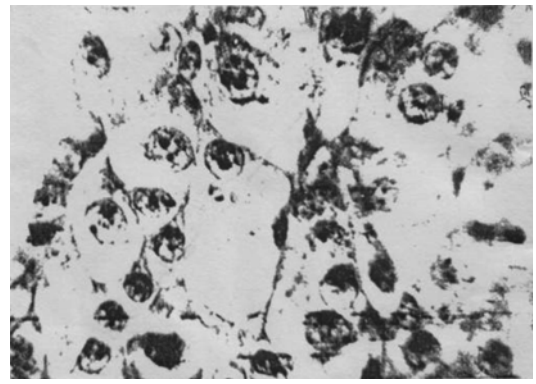

Fig. 10.7 A 'Titan cell' in the adrenal cortex; note the large size of the cell in comparison to others; × 1000

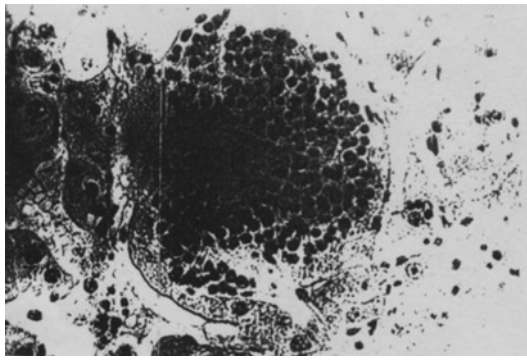

Fig. 10.6 High power view of the blood forming island of the fetal adrenal gland (14.5 g); note the erythrocytes in the sinusoids between rows of fetal cells. The blood forming islands are most developed in this period; × 400

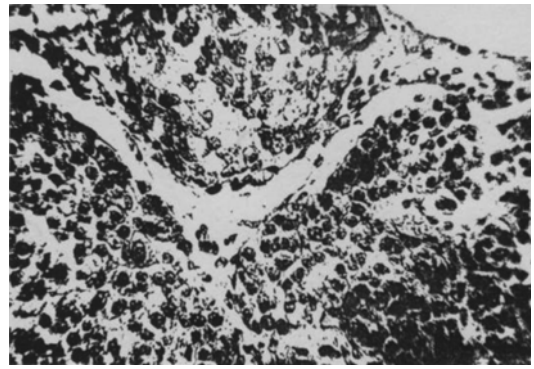

Fig. 10.8 Histological picture of the adrenal gland of a fetus weighing 25.4 g; note the indentation produced by a transmigrating medulla into the adrenal cortex pushing the adultogenic layer in front; × 100

At About 13 Weeks of Gestation

The histology of the adrenal gland of a fetus weighing 25.4 g and belonging to 13 weeks gestation is shown in Figs. 10.8 and 10.9. The capsule is well formed, contains three layers of cells. The adultogenic layer is of the same type as shown in the previous fetus. The fetal cells are big; towards the center of the fetal cells, the spaces between the cords of cells are occupied by sinusoids containing blood. The blood islands referred to previously are also there; they predominantly appear to be of the nature of a lymphoid follicle, naked intensely staining nuclei without much ctyoplasm; it is hard to be definite about the character of these cells.

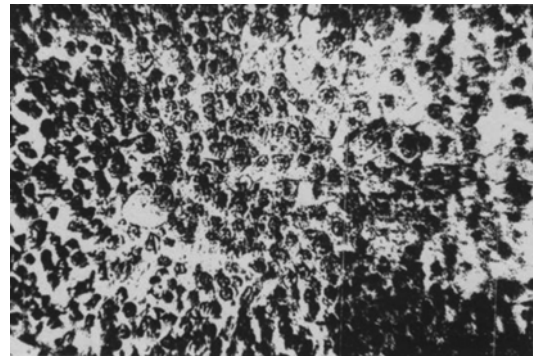

Fig. 10.9 Histological picture of the adrenal gland of a fetus weighing 25.4 g showing a 'Titan cell'; × 100

The most striking thing about the histology of this fetus was the demonstrability of the infiltration of medullary chords from the outside

through the capsule, through the adultogenic layer, and through the fetal layer towards the center of the gland. It appears that the medullary cells also form sort of islands which approach the capsule of the cortex and either the islands split the capsule by secreting hyaluronidase or the capsule sort of envelops the medullary islands, which then assume chord like structure and start to transmigrate.

At About 17 Weeks of Gestation

The histology of a fetus weighing 135 g and of 17 weeks gestation is shown in Figs. 10.10, 10.11, 10.12, and 10.13. The capsule is better formed than in the previous adrenal gland of 13 weeks. At places the connective tissue is more prominent and at places the cells are more prominent. What absorbs one's attention is the transmigration of the adrenal medulla. It appears as if the capsule splits into two layers to receive medullary tissue in its midst or,

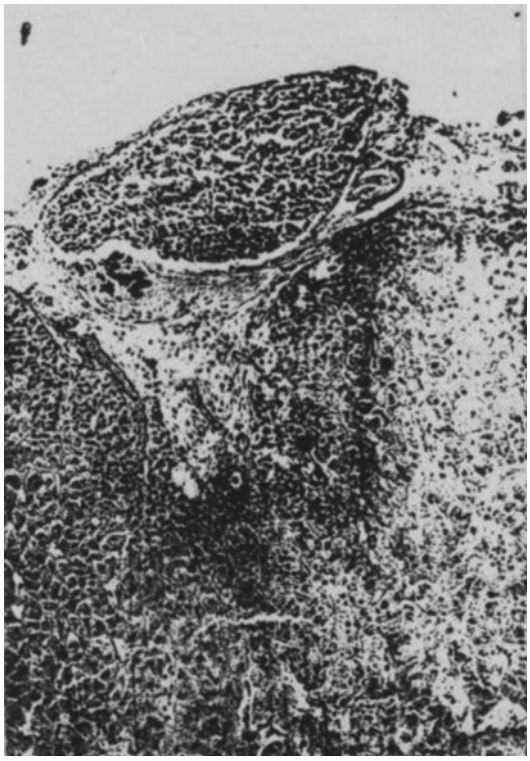

Fig. 10.10 Histological picture of the adrenal gland of a fetus weighing 135 g; the medulla is a rounded mass and is in the process of transmigrating into the cortex; the capsule splits to receive the medulla and the adultogenic layer is pushed inwards; × 100

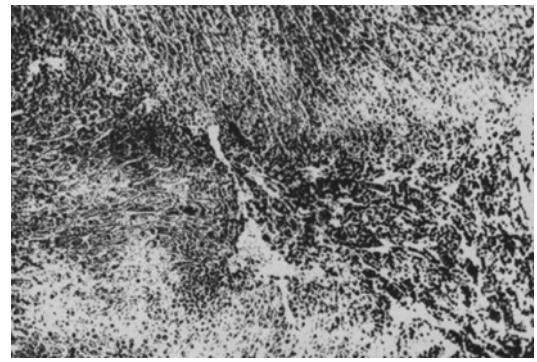

Fig. 10.11 Histological picture of the adrenal gland of a fetus weighing 135 g showing the details of the transmigration of the medulla; the rounded mass lengthens into strands and layers of medullary tissue going right through the adultogenic layer and the fetal cortex towards the centre of the gland; × 100

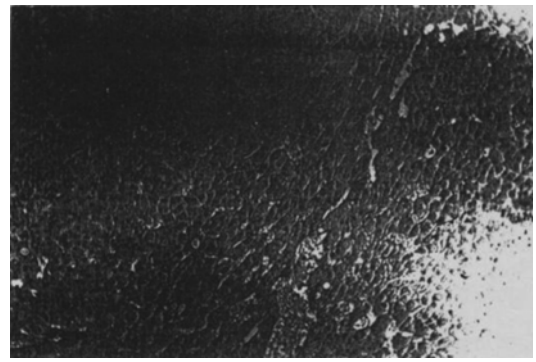

Fig. 10.12 Histological picture of the adrenal gland of 135 g in another area showing the ingrowth of a capillary in the wake of migrating medulla; × 100

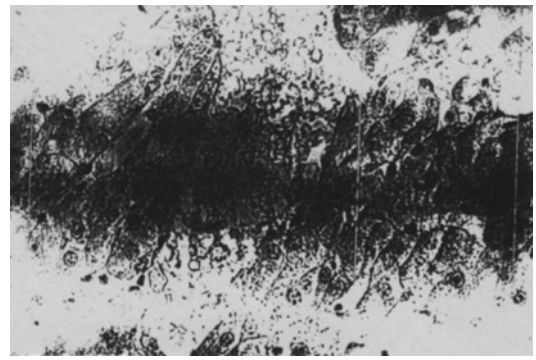

Fig. 10.13 A high power view of the capillary shown in Fig. 10.12

when the latter approaches the capsule, the capsule layer envelops and spreads a layer over it. The medullary tissue then produces an indentation on the inner layer of the capsulogenic mass, where the capsulogenic cells show signs of granulation (in PAS stained material). The adultogenic layer is bodily pushed aside by the transmigrating tissue (the original medullary tissue as it approaches the adrenal gland, begging to be included in the gland, has a capsule of its own. But as it penetrates the capsule and as it pushes the adultogenic layer, the capsule becomes disintegrated and the naked cells transmigrate). The capillaries follow in the wake of the path laid down by the adrenal medulla to go towards the center. At places they go by themselves without the help of the medullary tissue. At some places the actual capillaries have been cut in longitudinal section passing through the capsule and adultogenic layer into the fetal layer. As in the other glands, the adultogenic layer contains a few Titan cells. The fetal layer consists of large polyhedral cells, almost looking at you through microscope and begging you to unravel their mystery. The histology at this stage is a real beauty to see and to appreciate. The sinusoids are there containing blood. The islands of cells looking like lymphoid follicles are also there.

At About 19 Weeks of Gestation

The histological picture of the adrenal gland of a fetus of about 19 weeks gestation and weighing 240 g is shown in Fig. 10.14. The histological picture is a continuation of the process seen in the previous fetus as shown in Figs. 10.10 and 10.11. The capsule is at places permeated by blood vessels and the medulla, going to be incorporated in the substances of the adrenal gland. The adultogenic and fetal layers show the same general characteristics as shown in the illustrations of the previous fetus. The medullary tissue which has already gone in, forms a generally disorganized array of cells in the centre of the gland, and surrounded by fetal cortical cells.

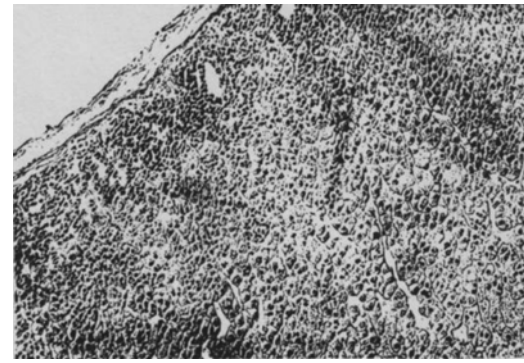

Fig. 10.14 Histological picture of the adrenal gland of a fetus of 19 weeks gestation, weighing 240 g: note the well formed capsules; the adultogenic layer is the same as in the earlier fetuses; the fetal cortex is well formed; the distinct sinusoidal lining contains erythrocytes, especially towards the centre of the gland; × 100

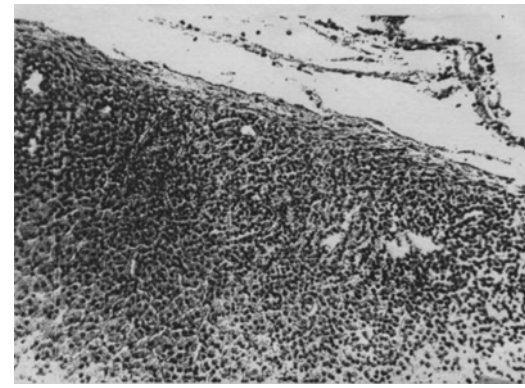

Fig. 10.15 Histological picture of the adrenal gland of a fetus weighing 635 g showing increased activity in the adultogenic layer and sinusoids in the adultogenic layer; × 100

At About 24 Weeks of Gestation

The histological picture of the adrenal gland of a fetus of about 24 weeks of gestation and weighing 635 g is shown in Fig. 10.15. The capsule, in some areas, consists of a few layers of connective tissue enclosing a large amount of loose areolar tissue. Included in the aredolar tissue are numerous blood vessels, arteries, veins and capillaries cut in cross sections. The incursion of medullary tissue is almost complete, i.e., in only one place in the section was an island of medullary tissue seen lying just outside the capsule.

The adultogenic layer is proliferating, and sinusoids are developing in this layer now. There are attempts at differentiation of this layer into a zona glomerulosa and zona fascicuata. The fetal cells are still good looking polyhedral cells containing sinusoids in between the chords. The medullary tissue is still a disorganized mass lying strewn in chord like structures within the fetal layer of cortical cells.

At About 28 Weeks of Gestation

The histology of the adrenal gland of a fetus weighing 1010 g and belonging to a gestation period of about 28 weeks is shown in Figs. 10.16 and 10.17. The capsule consists predominantly of fibrous tissue and contains in its midst a number of vascular channels, arteries, veins and capillaries. The adultogenic layer consists of incompletely formed zona glomerulosa and attempted formation of zona fasciculate. Strands of fibrous tissue dip down from the capsule separating the adultogenic layers into sort of lobules. The fetal cells still look pretty, healthy and well vascularized; the sinusoids are full of blood. Some wide vascular channels traverse the substance of the gland at places. The medulla could not be identified.

At About 32 Weeks of Gestation

Histology of the adrenal gland of a fetus weighing 1650 g and of about 32 weeks of gestation is shown in Fig. 10.18. The capsule is now fully differentiated. The adultogenic layer is similar to the layer in the earlier fetus shown in Figs. 10.12 and 10.13. The biggest change that has occurred is in the fetal layer. The cells no longer look that pretty; they have assumed a ground glass appearance, the nuclei have started to be pyknotic. Near the centre of the gland small lakes are forming in which amidst a large number of capillaries the fetal cells have started to degenerate. The degeneration is more severe in the centre than towards the peripaery.

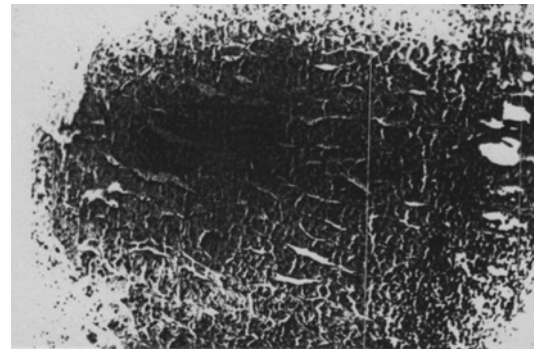

Fig. 10.16 Histological picture of the adrenal gland of a fetus, 28 weeks of gestation, weighing 1010 g showing increased activity in the adultogenic layer and necrosis of the fetal zone towards the centre of the gland; × 100

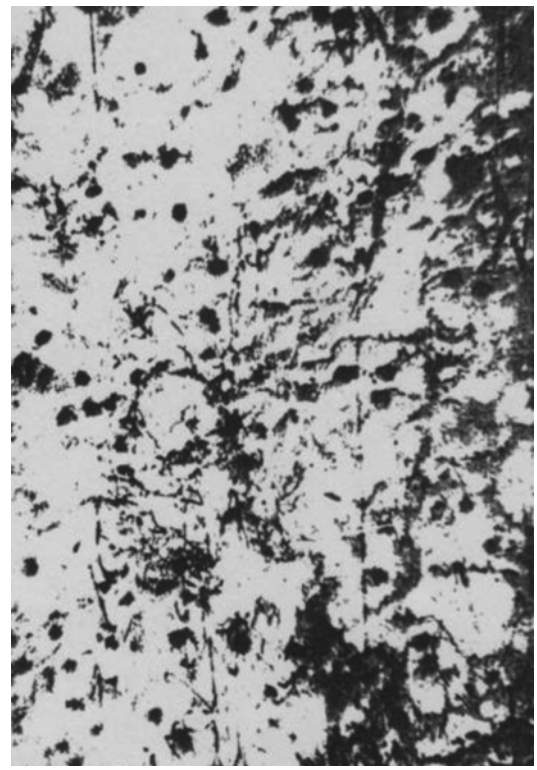

Fig. 10.17 Histological picture of the adrenal gland shown in Fig. 10.16, showing strands of fibres dipping down from the capsule towards the centre of the gland; × 400

At About 2 Days of Age

The histology of the adrenal gland of a child weighing 2500 g and who died of respiratory distress on the second day after birth is shown in

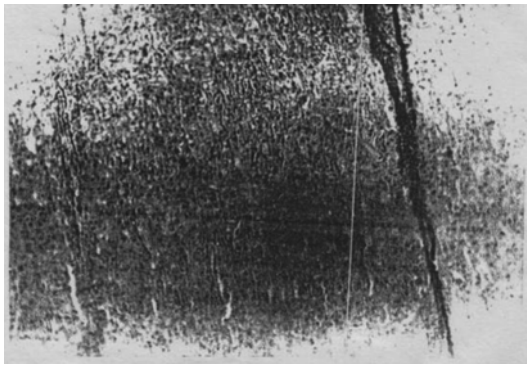

Fig. 10.18 Histological picture of the adrenal gland of a fetus weighing 1650 g showing vacuoles inside the fetal cortex; × 100

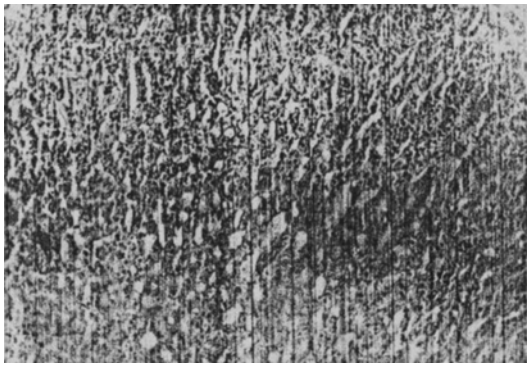

Fig. 10.19 Histological picture of the adrenal gland of a child of 2 years of age: note the tremendous proliferation of the adultogenic layer and the hyaline regeneration of the fetal layer; × 100

Fig. 10.19. The adrenal gland is still of the fetal type. The fetal layer is preparing to be degenerated and involuted. At places, especially towards the center of the gland there are a number of open spaces, possibly made by degeneration and disappearance of groups of fetal cells. The fetal cells which are remaining look sick.

At About 7 Days of Age

The histology of the adrenal gland of a premature child weighing 1100 g and who died 7 days after birth of cerebral hemorrhage (found in post mortem) showed that the gland was still a fetal gland. The adultogenic layer merged imperceptibly into the fetal layer which showed all signs of degeneration. Small islands were present where all the cells had autolyzed and were being removed. At places groups of cells had disappeared leaving tiny open spaces. But the whole process takes place so smoothly and efficiently that one cannot but wonder at the engineering feats of mother nature.

Fractionation of Soluble Proteins in the Adrenal Glands of Fetuses

When suprarenal glands of fetuses of different gestation periods were homogenized in buffered saline and centrifuged at 2000 × g for 15 min, the supernatants were found to contain proteins at a lower concentration in the fetuses of earlier gestation periods than in the fetuses of later gestation periods. One hundred microgram protein equivalent of these supernatants were separated by polyacrylamide gel electrophoresis and stained with amidoschwartz. From 10 to 15 different kinds of protein could be identified in the extracts of these fetuses; the pattern changed form one period of gestation to another; the change was quantitative as well as qualitative; i.e., new proteins appear, some old proteins disappear, others increase in intensity and some decrease. Their relationships to the functional differentiation of the gland as a whole are too complex to understand at this state of our knowledge but a start has been made. It is probable that some of these changes are associated with distinct functions like appearance of the different hydroxylases or in the later fetuses of the lysosmal enzyme. This change in the pattern of the soluble proteins continues through early postnatal life to adulthood.

Figure 10.20 shows the electrophoretic pattern of the soluble proteins of the adrenal cortex in fetuses weighing from 7 to 17 g, having gestation periods of 9–12 weeks. The line drawing of the pattern of one of these fetuses is shown in Fig. 10.21. The pattern was identical in these fetuses. Altogether eleven bands could be distinctly identified; they have been numbered form above downwards. The electrophoresis was run in such a way that the tubes were taken off just as the

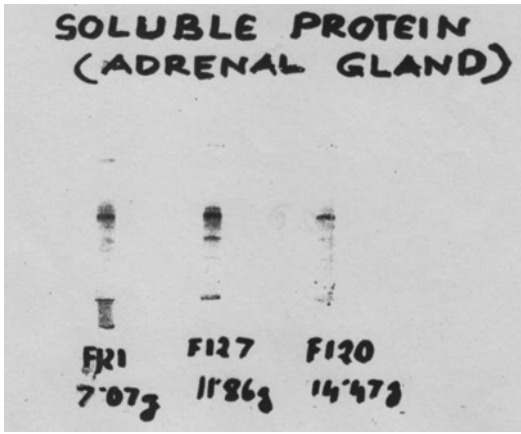

Fig. 10.20 The electrophoretic patterns of soluble proteins of the adrenal cortex in fetuses weighing from 7 g to 14 g, gestation periods 9 to 12 weeks

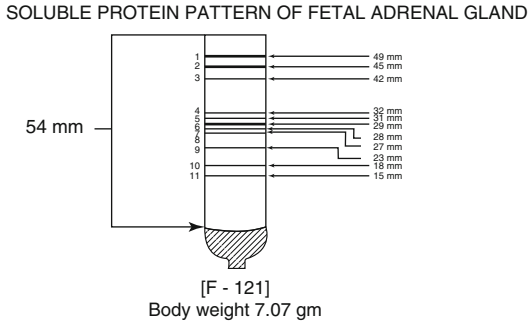

Fig. 10.21 Line drawing of soluble protein patterns of a fetus weighing 7.07 g, having gestation period of about 9 weeks approximately

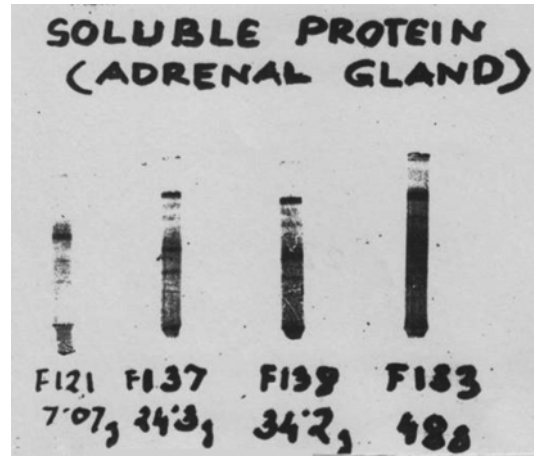

Fig. 10.22 The electrophoretic patterns of soluble proteins of the adrenal cortex in fetuses weighing from 24 to 48 g, gestation period 12–13 weeks; the pattern is compared to the fetus in Fig. 10.21

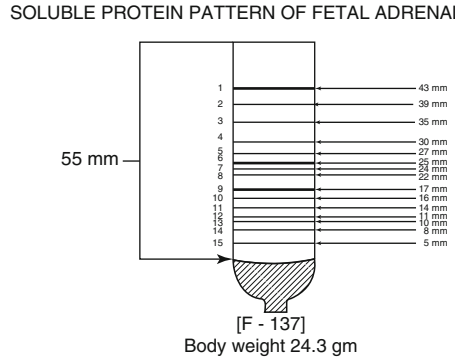

Fig. 10.23 Line drawing of soluble protein patterns of a fetus weighing 24.3 g, gestation period 12.6 weeks approximately

bromophenol blue dye band reached the lowermost point of the gel, so that the total length of the gel corresponded to the actual length of the column. The position of each band was then measured with a slide caliper and the distance of the band from the junction of the large and small pore gels divided by the total length of the gel was expressed as Rfs. Identical proteins in these bands were assumed to have the same Rfs. Figures 10.20 and 10.21 show that band No. 6 stained the most, followed by band 1 and 9 in decreasing order.

Figure 10.22 shows the electrophoretic pattern of soluble proteins of the adrenal cortex in fetuses weighing from 24 to 48 g, having gestation periods of 12–13 weeks. Altogether 15 bands could be observed. If we compare the pattern with that of the earlier stage, a profound change in pattern is certainly evident, i.e., 4 extra protein bands were present and the intensity of staining in band I increased and band 6 decreased. The pattern is compared to that of a the fetus shown in Fig. 10.23.

It can be speculated that one of these extra proteins can be the 21 hydroxylase which converts progesterone to deoxycorticosterone. The other three bands could be the other hydroxylases or some special proteins derived from the differentiating capsule or from the transmigrating medulla.

Figure 10.24 shows the electropolytic pattern of the soluble protein of the adrenal cortices in

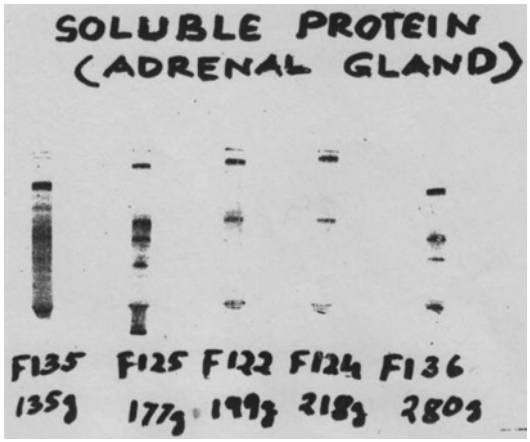

Fig. 10.24 The electrophoretic patterns of soluble proteins of the adrenal cortex in fetuses weighing 135–280 g, gestation period between 16 to 19 weeks

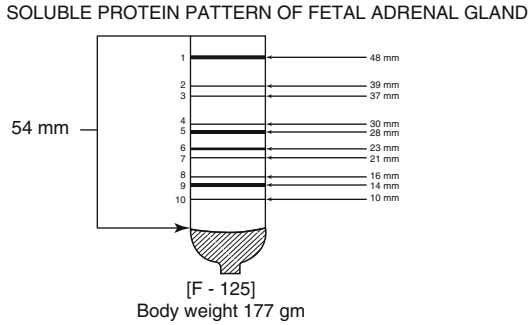

Fig. 10.25 Line drawing of soluble protein patterns of a fetus weighing 177 g, gestation period approximately 17.2 weeks

fetuses weighing from 135 to 280 g, having gestation period of 16–19 weeks. The line drawings of the same are given in Figs. 10.25, 10.26, and 10.27. The pattern shown in the fetus is more or less the same as the fetus shown in Fig. 10.22. But the pattern changes in the next fetus differing from it by only a few grams. In the pattern of soluble proteins, only 10 bands are discernible in contrast to 15 bands in the fetuses shown earlier. Four bands stain strongly 1, 5, 6 and 9, in contrast to 1, 6 and 9 in the fetus of later gestational age.

The banding patterns in the next three fetuses studied as exemplified in Fig. 10.28, shows more or less uniformity but the pattern again showed some change in fetuses that belonged to 20–24 weeks of gestation.

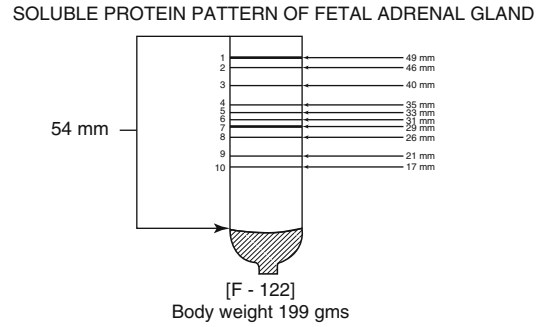

Fig. 10.26 Line drawing of soluble protein patterns of a fetus weighing 199 g, gestation period approximately 17.4 weeks

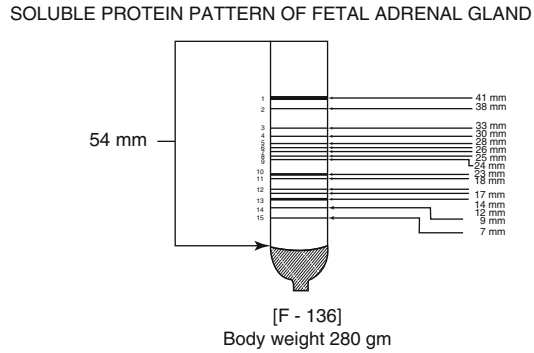

Fig. 10.27 Line drawing of soluble protein patterns of a fetus weighing 280 g, gestation period approximately 19 weeks

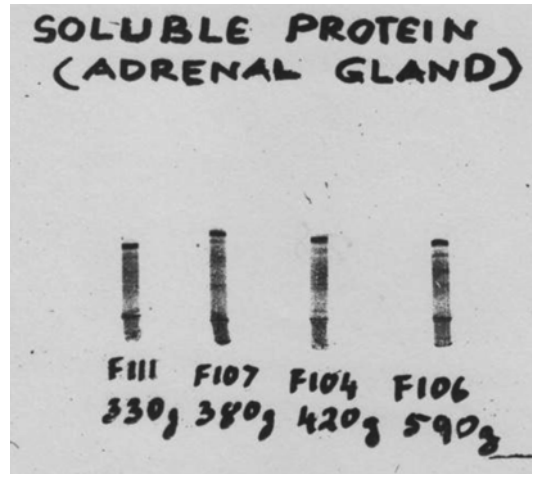

Fig. 10.28 The electrophoretic patterns of soluble proteins of the adrenal cortex in fetuses weighing 330 to 590 g, gestation period 20 to 24 weeks

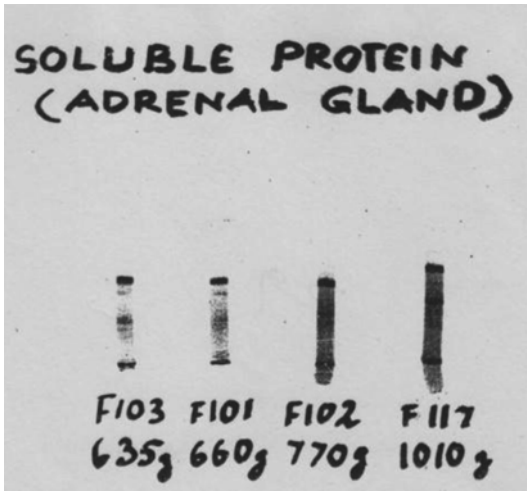

Fig. 10.29 The electrophoretic patterns of soluble proteins of the adrenal cortex in fetuses weighing 530 to 1010 g, gestation period 24 to 28 weeks

The banding patterns again changed in fetuses of 24 to 28 weeks gestation (Fig. 10.29).

The soluble protein pattern in postnatal life is exemplified in Figs. 10.30 and 10.31. The first column in the figure is from a baby who lived for 2 days and died of respiratory distress syndrome and the second column is from an adult person who died of a street accident. The number of bands in the two columns are the same but the individual staining intensity differed in the two cases. Altogether 24 bands were discernible in both of them. The most prominent difference between the two columns was with reference to bands 22 and 23. Whereas in the 2 day old baby the protein stained quite deeply, the staining was not pronounced in the case of the adult.

Fetal Specific Adrenal Protein

The change in the protein pattern of the developing fetal adrenal gland and comparing it with that of the adult adrenal gland suggested that the fetal gland probably contained some components which were not present in the adult gland and vice versa. So attempts were made to identify such proteins by immunological methods. The

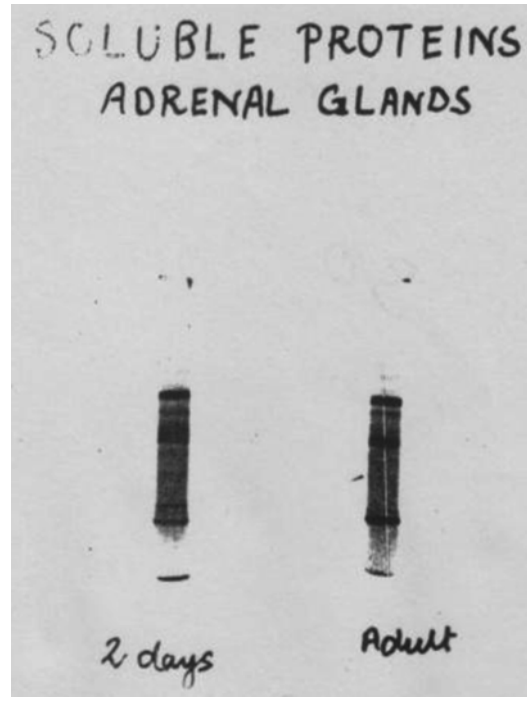

Fig. 10.30 The electrophoretic patterns of soluble proteins of the adrenal gland in post-natal life

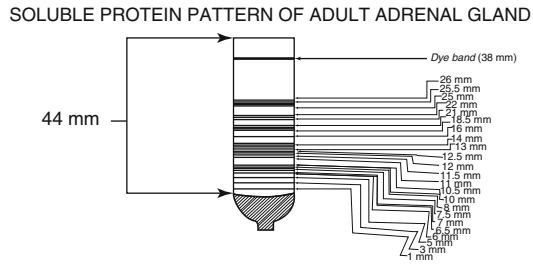

Fig. 10.31 Line drawing of soluble protein patterns of adult adrenal glands

fetal and adult cortical extracts were injected into rabbits in order to raise antibodies against the proteins present in the respective extracts.

Production of Rabbit Antibodies against Human Fetal and Adult Adrenal Cortical Extracts

Antisera were prepared by immunizing rabbits against (a) the soluble components, i.e., phosphate

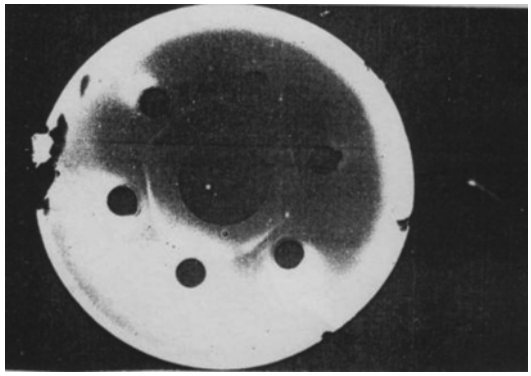

Fig. 10.32 The reaction pattern of antiserum to the phosphate buffered saline extract of fetal adrenal cortex (central well) with the phosphate buffered saline extract of the fetal adrenal gland (peripheral well) in Ouchterlony system

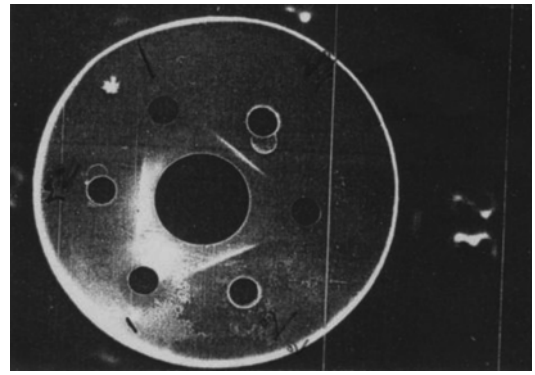

Fig. 10.33 The reaction pattern of antiserum to the phosphate buffered saline extract of fetal adrenal cortex (central well) with the phosphate buffered saline extract of the fetal adrenal gland (peripheral wells No. 2) and adult (peripheral wells No. 1) adrenal gland

buffered saline extracts of adrenal glands of both the fetus and adult and (b) membrane components, i.e., 1 M NaCl extracts of adrenal glands of both the fetus and adult, from which the soluble components were pre-extracted.

The extracts were injected along with equal volumes of Freund's adjuvants in order to have the maximum number of specific antibodies formed against the maximum number of proteins present in the extract. Not all proteins are equally immunogenic and fetal proteins are perhaps less immunogenic than adult proteins. Since a protein has to possess a structure, different from that of the corresponding protein of the host in order to behave as an antigen, many proteins of the human adrenal extract may possess structure identical with that of the rabbit and therefore will not behave as antigens. Even with these limitations in view the different antisera were compared with respect to their reactions with antigen chiefly by Ouchterlony technique [15].

Rabbit Antibodies to Fetal Adrenal Cortex (Phosphate Buffered Saline)

In the central well was placed the rabbit antibodies to the phosphate buffered saline extract of fetal adrenal cortex and in the peripheral wells, the phosphate buffered saline extract of the fetal adrenal gland, which was used to immunize the rabbits. Results are shown in Fig. 10.32. About five or six bands were discernible in 48 h as a result of the interaction, about two thirds of the distance away from the rim of the central hole.

When such antibodies were reacted alternately with phosphate buffered saline (PBS) extract of the fetal and adult adrenal glands, the reaction pattern was quite different in the two cases, as shown in Fig. 10.33. The reaction was read at the end of 24 h. The peripheral holes marked 1 contained adult PBS extract and those marked 2 contained fetal PBS extract. The reaction pattern with fetal PBS extract, of course, was similar to Fig. 10.32. About five bands could be observed, which, however, were closer to the central well than to the peripheral well, because the reaction was read at 24 h. The reaction pattern observed with adult PBS extract as shown in peripheral wells marked 1 was entirely different form that in wells numbered 2. Firstly, the reaction was much fainter; secondly, a number of bands were discernible which were identical with bands of wells numbered 2 but the bands travelled more towards the peripheral hole than the bands in wells numbered 2; thirdly a faint reaction could be observed with another set of antigens in the adult PBS extract.

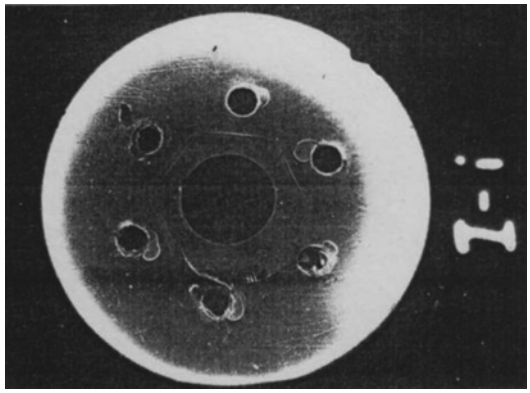

Fig. 10.34 The reaction pattern of antiserum to 1M NaCl extract of fetal adrenal gland (central well) with 1M NaCl extract of fetal adrenal glands (peripheral wells)

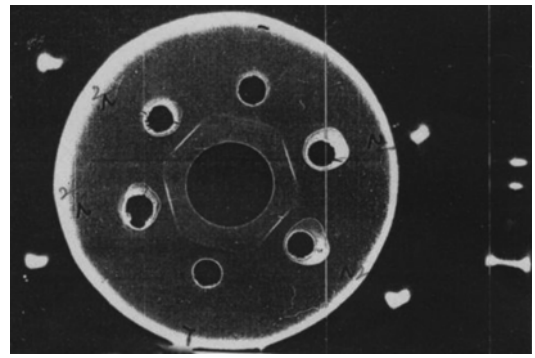

Fig. 10.35 The reaction pattern of antiserum to 1M NaCl extract of fetal adrenal gland (central well) with 1M NaCl extract of fetal and adult adrenal cortices; peripheral well No.1 contained fetal 1M NaCl extract and peripheral well No.2 contained adult 1M NaCl extract

Antibodies to Fetal 1M Extract of Adrenals

Rabbit antibodies to 1M NaCl extracts of fetal adrenal glands were placed in the central well and were allowed to react with 1M NaCl extract of the fetal adrenal gland which was used to immunize the rabbits. The pattern of reactions is shown in Fig. 10.34. Two groups of antigens were discernible, one group having smaller molecular weights on the average than the other. The group having the higher molecular weights was present in a greater concentration than the group having the smaller molecular weights.

When such antibodies were reacted alternately with 1M NaCl extracts of fetal and adult adrenal cortices, the reaction pattern was again different (Fig. 10.35). Peripheral wells marked 1 contained 1M NaCl extract of the fetal adrenal gland and those marked 2 had 1M NaCl extract of the adult adrenal gland. The reaction in the holes marked 1 showed identical pattern as shown in Fig. 10.34, i.e., two sets of proteins were discernible; but in the holes marked 2, only one set of proteins was discernible with showed reactions of identity with the group of antigens of fetal 1M extracts having the higher molecular weights. No proteins were found in the adult 1M adrenal extract corresponding to the second set of proteins of the fetal 1M adrenal extract.

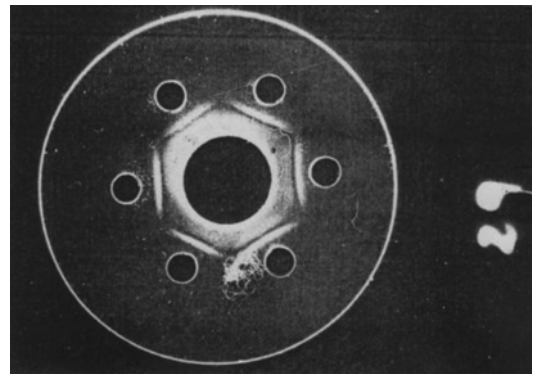

Fig. 10.36 The reaction pattern of antiserum to PBS extracts of adult adrenal gland (central well) with same PBS extracts of adrenal gland (peripheral wells)

Antibodies to PBS Extracts of Adult Adrenal Glands

Rabbit antibodies to PBS extracts of adult adrenal glands were placed in the central hole and were reacted with the same PBS extracts of adrenal glands which were used as the immunogen. Results are shown in Fig. 10.36. Two broad groups of protein were discernible in the reaction pattern, separated from each other by a clear zone. These two groups of protein had again differing molecular weights, one group having a group average molecular weight more than the other.

When such antibodies were reacted alternately with PBS extracts of fetal and adult adrenal

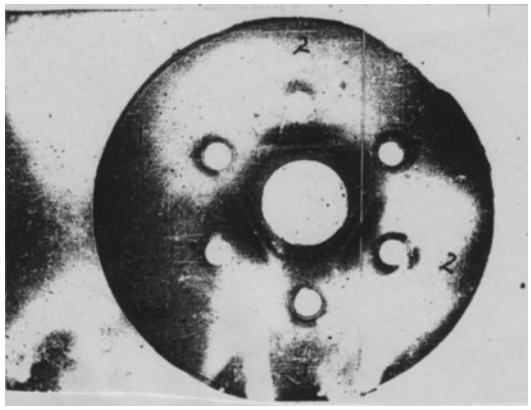

Fig. 10.37 Same antiserum reacted alternately with PBS extracts of fetal (Hole No. 1) and adult (Hole No. 2) adrenal glands

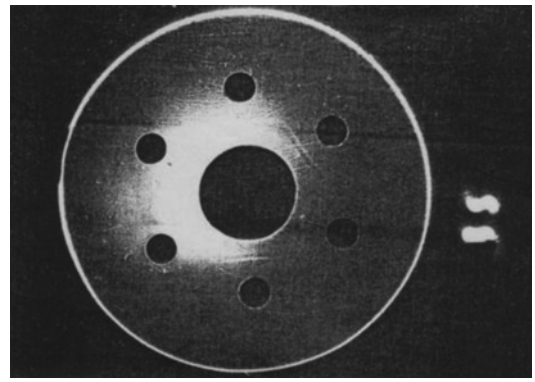

Fig. 10.38 The reaction pattern of antiserum to 1M NaCl extracts of adult gland (central well) with corresponding antigen (peripheral wells)

glands, results are shown in Fig. 10.37. Wells marked 1 had extract of fetal glands and two contained extracts of adult glands. The reaction with the fetal extracts was much fainter than the reaction with adult extracts. Only one group of proteins were discernible, this group giving reactions of identity with the group in the adult cortical extract, the inner group.

The reaction with the adult PBS extract, however, revealed two seats of bands as in Fig. 10.36; the external set was specific for the adult PBS extract in the sense that no corresponding bands could be found in the fetal PBS extract. The inner set of bands, as stated previously showed reactions of identity with the fetal PBS extract; however it stained more intensely than the corresponding bands of the fetal PBS extract. It appeared as if the inner set with the adult PBS extract contained more bands than with the fetal PBS extract but since the corners were well rounded it appears that these sets of bands were identical.

Antibodies to 1M NaCl Extracts of Adult Adrenal Glands

Rabbit antibodies to 1M NaCl extracts of adult adrenal glands were placed in the central well and the corresponding antigen was placed in the peripheral wells. Results are shown in Fig. 10.38. Two sets of bands were discernible one external and the other internal, differing in average molecular weights with a gap in between.

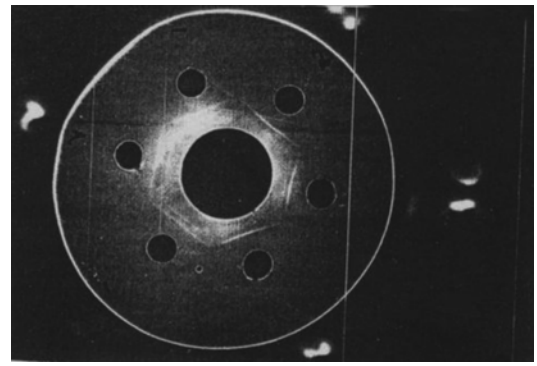

Fig. 10.39 Same antiserum reacted with 1M NaCl extracts of fetal (Hole No. 1) and adult (Hole No. 2) adrenal gland

When such antibodies were reacted with 1M NaCl extracts of fetal (hole 1) and adult (hole 2) adrenal glands, the reactions were as shown in Fig. 10.39. Two sets of bands were observed in each case and they were identical.

Antibodies to PBS Extracts of Fetal Adrenal Glands

When rabbit antibodies to PBS extracts of fetal adrenal glands were placed in the central hole and reacted with 1M NaCl extracts of either fetal (hole 1) or adult (hole 2) adrenal glands, results are shown in Fig. 10.40. Almost no precipitating bands were detectable in either case. This shows that PBS extracted antigens were different from

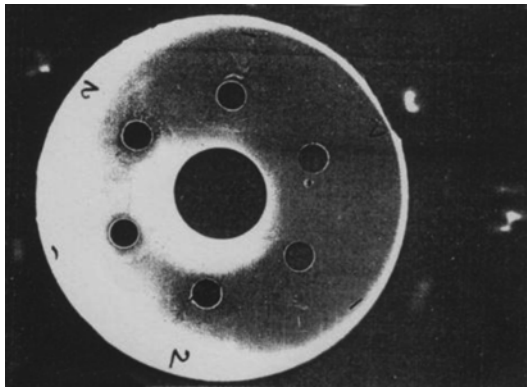

Fig. 10.40 The reaction pattern of antiserum to PBS extracts of fetal adrenal gland (central well) with 1M NaCl extracts of fetal (hole 1) and adult (hole 2) adrenal glands

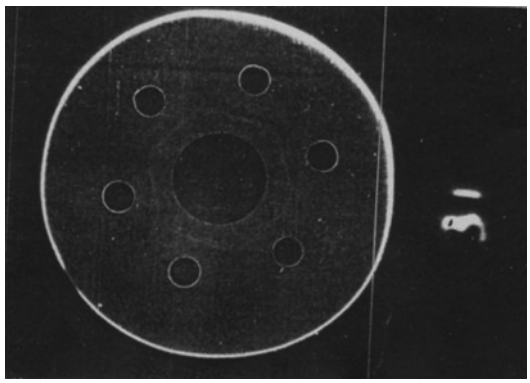

Fig. 10.41 The reaction pattern of antiserum to PBS extracts of adult adrenal gland (central well) with 1M NaCl extracts of adult adrenal gland (peripheral holes)

the 1M NaCl extracted antigens (compare Fig. 10.40 with Fig. 10.33.)

Antibodies To PBS Extract of Adult Adrenal Gland

Rabbit antibodies to PBS extract of adult adrenal glands were placed in the central well and reacted with 1M NaCl extract of adult adrenal glands in peripheral wells. The reaction is shown in Fig. 10.41. Two groups of antigens were discernible. It appears, therefore, that PBS extracted antigens in the case of adult glands had common antigens with the 1M NaCl extracted antigens. When such antibodies were reacted with 1M NaCl extracts of fetal glands, results were

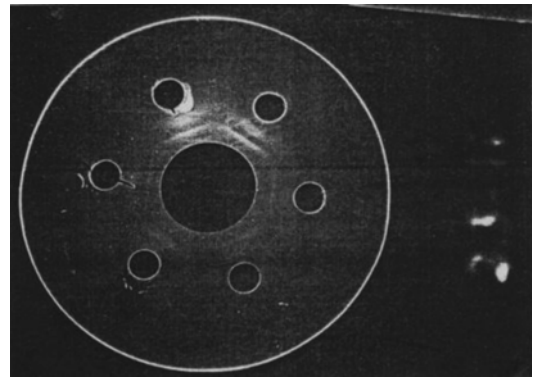

Fig. 10.42 The reaction pattern of antiserum to PBS extracts of adult adrenal gland (central well) with 1M NaCl extracts of fetal adrenal gland (peripheral wells)

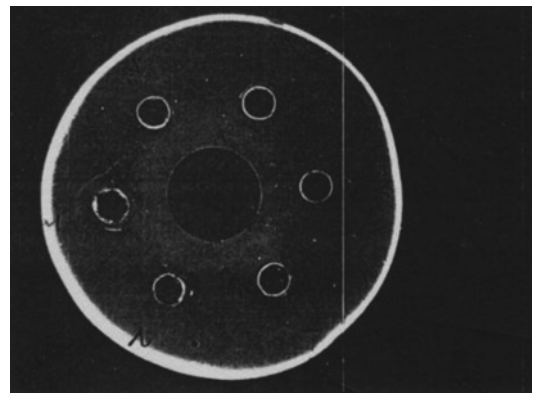

Fig. 10.43 The reaction pattern of antiserum to 1M NaCl extracts of adult adrenal glands (central hole) with PBS extracts of adult (hole 1) and fetal adrenal (hole 2) adrenal glands. Near Hole 1, arrow indicates the antigen antibody precipitin band

obtained as in Fig. 10.42. The results are identical with the results of Fig. 10.41.

Antibodies to One M Nacl Extracts of Adult Adrenal Glands

When rabbit antibodies to 1M NaCl extracts of adult adrenal glands were extracted with PBS extracts of adult (hole 1) and fetal (hole 2) adrenal glands results are shown in Fig. 10.43. With adult PBS extracts (hole 1), three sets of bands were discernible. The photographic reproduction is poor. But in the actual plate two sets of antigen antibody reactions were seen very close to the antigen hole (shown by the arrow); these were

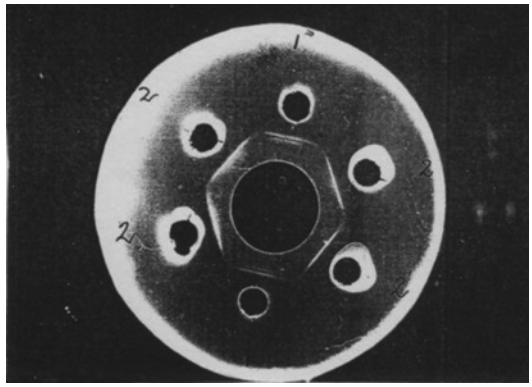

Fig. 10.44 The reaction pattern of antiserum to 1M NaCl extracts of fetal adrenal gland (central hole) with 1M NaCl extracts of fetal (peripheral well no. 1) and adult (peripheral well no. 2) adrenal cortices

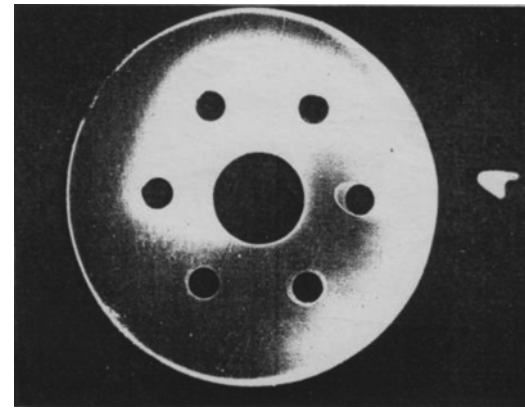

Fig. 10.45 The reaction pattern of the supernatant (central well) from the precipitated antibody with the 1M NaCl extract of adult adrenal gland (peripheral wells)

not present near hole, 2 i.e., with fetal PBS extracts. The third set of bands was near the central hole and consisted of a broad band, not much well differentiated. Near hole 2, i.e., with fetal PBS extracts, two sets of bands were observed; the inner set was identical with the innermost bands of the adult PBS extracts but the second set of bands was characteristic of fetal PBS extracts.

Characterization of Fetal 1M Antigens

Figure 10.44 shows the reaction of rabbit antibodies against fetal 1M antigens with fetal (hole 1) and adult (hole 2) 1M antigens. As noted before, the reaction with fetal 1M extract showed two sets of bands, one of which was absent in the reaction with corresponding adult antigen in hole 2. One set of antigens was, therefore, common to fetal and adult 1M NaCl extracts but one set of antigens were characteristic of fetal 1M NaCl extracts. It was, therefore, considered worthwhile to study if such fetal specific antibodies could be characterized. Accordingly 1 ml of the rabbit antibodies was incubated with 0.1 ml of the adult 1M NaCl extract at 4 °C for 18 h after which the mixture was centrifuged; the supernatant was again treated with 0.1 ml of the adult 1M NaCl extract for 18 h at 4 °C and recentrifuged; the process was repeated for two more times; at the end of which time the antibodies did not show any reaction when reacted with 1M NaCl extract of adult adrenal glands as shown in Fig. 10.45.

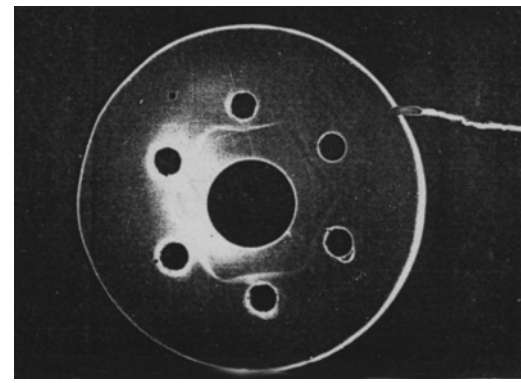

Fig. 10.46 The reaction pattern of the same supernatant (central well) with 1M NaCl extracts of fetal adrenal gland (peripheral wells)

The central well contained the supernatant from the precipitated rabbit antibody and the peripheral wells contained the 1M NaCl extract of adult adrenal glands. Although the rabbit antibodies by such a treatment did not react with 1M NaCl extracts of adult adrenal gland they still showed reaction with 1M NaCl extracts of fetal adrenal glands. Figure 10.46 shows the reaction pattern when such a supernatant was placed in the central well and the 1M NaCl extracts of fetal adrenal glands in the peripheral wells. It shows that 1M NaCl extracts of fetal adrenal glands contained some immunogenic components which were probably not present in the extracts from adult adrenal glands.

Presence of Autoantibodies in Postnatal Life against Fetal Adrenal Antigens

Ouchterlony System

If the fetal cortical cells possessed a soluble antigen, which was not present in the adult type of cortical cells, it may be possible that such antigen might give rise to humoral antibodies in postnatal life. It was presumed, of course, that in antenatal life, the synthesis of such antibodies was inhibited, possibly by placental hormones.

An infant, especially in the first few months of life, is active in the involution of fetal cortical cells. If the involution is due to complement mediated antigen antibody reaction, the serum of an infant is most likely to show the presence of such antibodies. The serum of a number of infants aged between 4 and 12 months was placed in the central well of an Ouchterlony system and fetal cortical extracts both with phosphate buffered saline and with 1M NaCl solution were placed in the peripheral wells. Even in a 1 week old infant, no precipitin band was found to develop. Figure 10.47 shows one such experiment. No antigen antibody precipitating band was demonstrable.

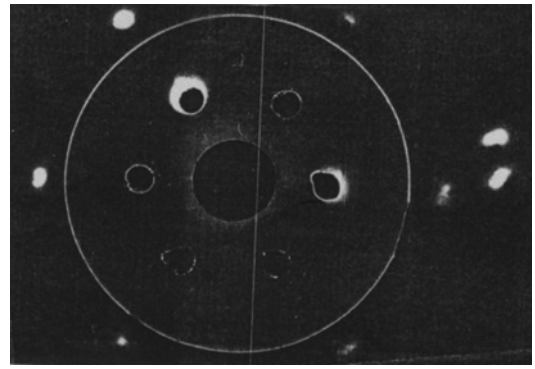

Fig. 10.47 The reaction pattern of infant serums (central well) with fetal cortical extracts both with phosphate buffered saline (peripheral hole no. 1) and with 1M NaCl solution (peripheral hole no. 2) in Ouchterlony system

Table 10.1 Precipitin reaction infant serum with adrenal extracts

Infant serum dilution	Fetal extracts/protein mean difference i.e. ppt. (μg)			Child extracts/protein mean difference i.e. ppt. (μg)		
Control	14.8	11		41.44	42	
1/5	34.60	47	36	61.45	53	11
1/10	28.46	37	26	33.16	24	0
1/20	7.11	9	0	44.31	37	0
1/40	16.26	21	0	40.40	40	0
1/50	13.14	13	0	38.44	41	0

Quantitative Immunoprecipitation Test

The presence of certain specific components in the fetal adrenal extract in contradistinction to adult adrenal extract invited some studies on the humoral and cell mediated immune response of infant to the fetal cortical cells. No frank autoantibodies could be demonstrated in the serum of infants who were active in the involution of the fetal cortical cells, to the fetal adrenal extracts. *In vitro* study of quantitative Immuno Precipitation test was done by using infants serum at various dilution and either fetal cortical extract or a child's cortical extract. Results are shown in Table 10.1. When infant serum as different dilutions was mixed with fetal cortical extracts, measureable precipitate could be obtained up to a serum dilution of 1 in 10. Under identical conditions almost no precipitate was noted when the same sera were mixed with adrenal extract of children. Whether the precipitate obtained with the fetal cortical was real or not is a problem for discussion. As can be seen from the table, the protein content in the control tube with fetal cortical extract was less than that in the control tube with child's adrenal extract. It may be that the proteins of the fetal cortical extract were more soluble than those of the child's adrenal extract and did not precipitate so much as a result of incubation at 4 °C for 96 h. But the increase of precipitate when infant's serum was added to the fetal cortical extract was real and consistently found in four consecutive experiments, of which one is shown in Table 10.1.

Fifty μl of saline extracts of the adrenal cortices from the fetus and a 1 year old child, containing 100 μg protein was mixed with 50 μl of the appropriate dilutions of the infant serum. The mixture was incubated at 4 °C for 96 h, and centrifuged; the precipitates were once washed with cold saline; protein was then estimated directly in

the tube. From the protein contents of the experimental tubes, that of the control tube were subtracted and results are expressed as the difference from the control.

Passive Hemagglutination Test

When sheep erythrocytes were either tanned or trypsinized and then coated with fetal and adult cortical PBS extract, it was found that the erythrocytes to which the fetal cortical extract was adsorbed, could be agglutinated by infant serum up to a dilution of 1 in 20, but not by fetal serum or adult serum. No hemagglutination was observed with erythrocytes coated with adult adrenal PBS extract. Results are shown in Table 10.2, against fetal serum, infant serum and adult serum dilutions the results were consistent in six experiments, one of which is reported in Table 10.2.

The results of passive hemagglutination test with both the fetal and adult adrenal extracts against different dilutions of infant, fetal and adult sera are given.

Passive Cutaneous Anaphyl Axis Test

We next tested the assumption that the infant in whom the involution of fetal adrenal cells is taking place, may manifest some cell mediated immune response to the fetal cortical cell, provided some immunological mechanism is at the basis of the involution. Accordingly fetal and adult adrenal extract (both saline and 1M NaCl extracts) were made up to the same protein concentration of 40 µg per 10 µl and then sterilized by passing through Millipore filter. Such extracts were sterilized by passing through millipore filter. Such extracts, however, when injected intradermelly into a number of infants failed to produce any measurable induration. An Arthustype reaction also could not be demonstrated with such adrenal extracts. The infants were injected with Even's blue 15 min prior to the intradermal injection of the adrenal extracts. No bluish discoloration could be observed at the site of injection (Fig. 10.48).

Migration Inhibition Factor of Sensitized Lymphocytes and Macrophages

Leucocytes were prepared form the blood of a child aged 10 months and who was tuberculin positive; they were cultured in Lucite Kennedy Chambers in the presence and absence of fetal cortical extracts and tuberculin. Results are shown in Fig. 10.49. In chamber Nos. 1 and 2, the leucocytes were cultured without any antigen. The area of leucocyte migration was measured by plainmetry and found to be 2.8 and 3.2 mm^2 respectively. In Chamber Nos. 3 and 4, the medium contained 6 µg of protein equivalent of fetal cortical extract per ml of medium, there was complete inhibition of migration. Since the child was tuberculin positive, Chamber Nos. 5 and 6 contained medium to which was added 1 µl of PPD. In this case too, there was complete inhibition.

Table 10.2 Passive hemagglutination test

Reaction with fetal serum								
Infant serum dilution	1/5	1/10	1/20	1/40	1/80	1/160	1/320	Control
Fetal Ext.	+	+	+	–	–	–	–	–
Child Ext.	–	–	–	–	–	–	–	–
Reaction with fetal serum								
Fetal serum dilution	1/5	1/10	1/20	1/40	1/80	1/160	1/320	Control
Fetal Ext.	–	–	–	–	–	–	–	–
Child Ext.	–	–	–	–	–	–	–	–
Reaction with adult serum								
Adult serum dilution	1/10	1/20	1/40	1/80	1/160	1/320	Control	
Fetal Ext.	–	–	–	–	–	–	–	
Child Ext.	–	–	–	–	–	–	–	

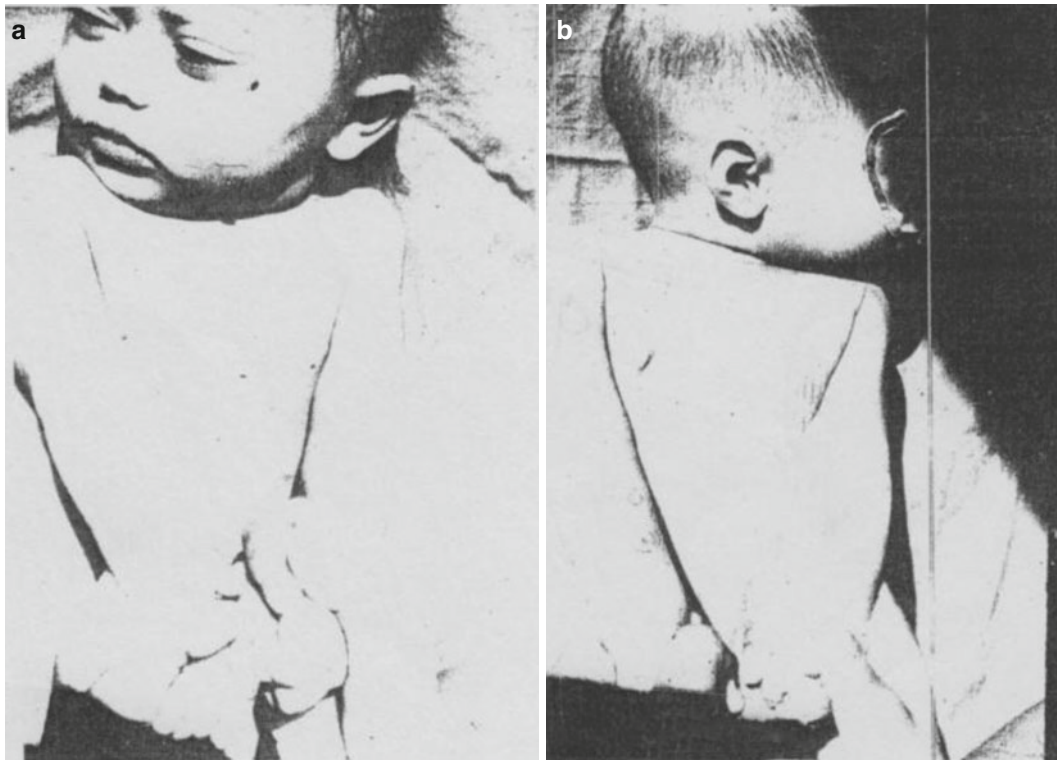

Fig. 10.48 Passive outaneous anaphylaxis reaction in infants, 48 hours after intradermal injection of extracts; 2 ml of 2 % Evan's blue solution (50 gm/kg) was injected intradermally prior to the administration of the extract: (**a**) PBS extracts of both the fetal and adult adrenal glands were injected intradermally on the left and right forearms of the infant; (**b**) 1M NaCl extracts of both fetal and adult adrenal glands were injected intradermally on the left and right forearms of the infant. A control site injected with saline is also marked on both. No reaction can be seen in either case

When leucocytes were prepared from an adult blood in the same way and then cultured in the presence and absence of adult and fetal cortical extracts, the migration observed is also depicted in Fig. 10.49. Whereas in the control Chamber Nos. 7 and 8, the area of leucocyte migration amounted to 3.0 and 2.5 mm^2 respectively, there was complete inhibition of migration in the presence of fetal cortical extract (Chamber Nos. 10 and 11) and tuberculin (Chamber No. 12) in the presence of adult cortical extract, however, the leucocyte migration was not completely inhibited (Chamber No. 9). The area of migration was found to be 1.6 mm by plainmetry.

There was complete inhibition of infant leucocyte migration with as little as 6 μg of protein equivalent form fetal cortical extract. Thus the leucocytes of infants may be presumed to be sensitized to the fetal cortical extract. What part it plays in the death of these cells and their involution we cannot say.

Conclusion

We have studied histologically the developmental pattern of human fetal adrenal cortex from early gestation period towards the end of gestation period (3–32 weeks of gestation), infants, children, adult. Histologically the human fetal adrenal cortex was different from adult adrenal cortex. The two kinds of cells are probably distinct to their derivation too in fetal adrenal cortex. On the periphery of glands deep stained, there are a few layers of cells called "Permanent zone" or "Adult zone" and the majority of the gland is

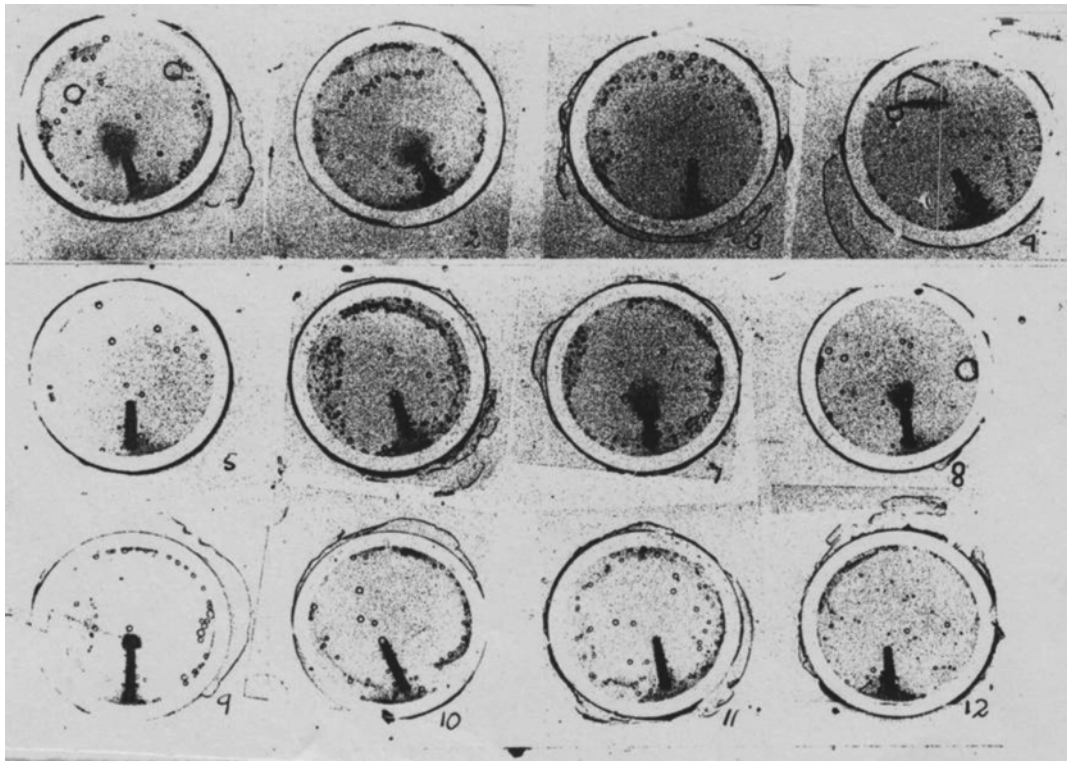

Fig. 10.49 Inhibition of sensitized lymphocytes migration. 1–6: lymphocytes from a 10 month old child who was tuberculin positive exhibiting sensitization to fetal adrenal cortical extract (*1*) and (*2*) without any antigen; the area of migration is 2.8 and 3.2 sq. mm; complete inhibition of migration has been exhibited in chamber *3*. *4*, the medium containing fetal cortical extract, and in chamber (*5*) and (*6*), the medium containing PPD. &-12: containing lymphocytes from an adult tuberculin-positive person; *7* and *8* – without any antigen: the area of migration is 3.0 and 2.5 sq. mm; (*9*): the area containing adult cortical extract: the area of migration is 1.6 sq. mm; complete inhibition of migration has been exhibited in (*10*) and (*11*) – the medium contained fetal cortical extract; (*12*) contained PPD

occupied by the large polyhedral cells which are frequently fat containing and have no definite arrangement; this portion is called "Fetal zone". In the most central region of the gland a few deeply staining medullary cells may be seen. The involution of the fetal cortical cells, which starts in the last couple of weeks of gestation and continues through postnatal life is associated with death of the fetal cells. In adult adrenal gland no fetal zone is found and adult zone proliferates into three zones, i.e., Zona glomerulosa, Zona fasciculate and Zona reticilaris.

Besides their distinctive histological characters, can the fetal and adultogenic layer be distinguished by any other characteristics? The soluble proteins of the fetal adrenal glands of different gestation period was studied by Polyacrylamide gel electrophoresis method and stained by amidoschwartz from 10 to 15 different kinds of protein could be identified in the extracts of the fetuses; [19] the pattern change from one period of gestation to another, Some old proteins disappear, new proteins appear, other increase in intensity and some decrease. The majority of the workers believe that the principal steroidogenic cells are the fetal, while some specialized function like formation of pregnenolone and 11-β- OH-androsteredion by the whole adrenal gland is carried on [16].

Besides their distinctive histological characters, can the fetal and adultogenic layer be distinguished by any other characteristics? Since the

functional characteristics of the two kinds of cells also differ, can we identify any protein or other component present in the fetal layer of cells which is absent in the adult layer? Accordingly, we prepared saline and 1M NaCl extracts of fetal and adult adrenal glands. The saline extracts of course contain besides cytosol, the mitochondria, endoplasmic reticula, golgi bodies and other particulate matter. The 1M NaCl extracts contain nuceloprotiens, glycoprotenis, membraneous semisoluble proteins and the like.

These two kinds of extracts from the fetal and adult adrenal glands were injected into rabbits with Freund's adjuvants in order to raise antibodies against antigens present in the different extracts. These antibodies were, then, reacted against these extracts *in vitro* in Ouchterlony systems. When antibodies against saline extracts of fetal adrenal glands were reacted with saline extracts of fetal and adult adrenal glands, the reaction pattern showed that in addition to common antigens, the adult saline extract contained some antigens which were not present in the fetal saline extract.

When antibodies to 1M NaCl extracts of fetal glands were reacted with 1M NaCl extracts of fetal and adult adrenal glands, some components in the fetal and adult extracts reacted in common, the reaction pattern with the common antigens were reactions of identity. There was a separate set of antigens in the 1M NaCl extracts of fetal glands; these antigens were not present in the adult adrenal gland. The findings of some fetus specific component in the 1M NaCl extracts of fetal glands, therefore, assumes some importance.

When rabbit antibodies were raised against saline extracts of adult adrenal glands and such antisera were tested with saline extracts of adult and fetal adrenal glands, the antigen antibody reactions were more intense with adult extract than with fetal extract. Furthermore, a set of antigens were found in adult extracts which were not present in fetal extract. It can be inferred that either the adult saline extract does not have some common fetal antigens or the differentiation of the adult adrenal gland is associated with the appearance of a host of specific antigens.

In the case of antibodies to 1M NaCl extracts of adult adrenal glands, however, the immunogenic components in the corresponding adult and fetal extracts were similar.

The antibodies to saline extracts of the fetal gland did not react with 1M NaCl extracts of either fetal or adult glands. So the components extracted with saline and 1M NaCl were antigenically distinct and specific.

The antibodies to saline extracts of the adult gland reacted with 1M NaCl extracts of the adult as well as fetal glands. In the case of adult glands, therefore, the saline extracted components had common antigens with 1M NaCl extracted antigens.

Rabbit antibodies against 1M NaCl extracts of the fetal glands showed one set of precipitating bands which was common to 1M NaCl extracts of both fetal and adult adrenal glands and one set of bands which was present with corresponding fetal extract only. The antibodies to the immunogens, which were common to 1M NaCl extracts of adult and fetal glands could be precipitated with 1M NaCl extracts of adult glands. Such serum no longer reacted with 1M NaCl extract of adult glands but still reacted with 1M NaCl extracts of fetal glands. The fetal glands, therefore, had perhaps, located on the cell membrane, some immunogenic components, which were unique to the fetal cells and not present in either the adultogenic layer of the fetal gland or the differentiated cells of the adult gland.

Can this unique fetal component on the membrane of the fetal cortical cell serve as a heterologous antigen, which the immunologic apparatus of the older fetus and infant recognize as a foreign component and so it proceeds to reject the cells containing the foreign components? So it was considered worthwhile to investigate whether the new born or infant serum contains some humoral antibodies to soluble extract of the fetal adrenal gland, or whether the lymphocytes or macrophages of the infant can produce cell mediated immune response against fetal adrenal component.

When untreated sera of new-borns and infants were reacted with either saline or 1M NaCl extracts of fetal adrenal glands in an Ouchterlony

system, no precipitating band could be demonstrated. Even when γ-globulins were precipitated with salts and such γ-globulins were dialyzed and concentrated and then use the system, no humeral antibody could be detected.

The immunoprecipitin test as well as the passive hemagglutination test with coated tanned erythrocytes show the presence of some sort of humoral antibodies to the fetal cortical extract in infant serum, although the reaction in either case was not pronounced one. As a matter of fact, it was not expected that the reaction would be a very pronounced reaction considering that the involution of the fetal cortex takes place over a matter of months in a slow and gradual way, rather than like the involution that occurs in the uterus after parturition which can be regarded as an explosive event.

The humoral reaction, however, cannot by itself account for the ordered progression of death and involution of the fetal cortical cells. It was considered worthwhile to test of the presence of cell mediated immune response of infants to fetal cortical extract. However, when both kinds of extracts of fetal and adult adrenal glands were injected intradermally into the forearm of 5, 6 month old infants, no induration could be detected 2, 4, 24 and 48 h after the injection. No Arthus type of reaction was also demonstrable in as much as there was no discoloration at the site of intradermal injection when such infants were preinjected with Evan's blue (T 1824).

If we presume that the fetal membrane component of fetal cortical cells is unique to fetal cells, and that this component is recognized by the involuting individual as foreign to the organism, we can expect the immunocompetent cells to react to it. Such reaction may take various forms, such as migration inhibition factor, skin reactive factor, lymphotoxin, inhibitor of DNA synthesis, chemotactic factor, blastogenic factors etc. [17].

The leucocytes will be sensitized to the fetal component and show disapproval on coming in contact with fetal adrenal extract. One of the ways in which disapproval may be manifested is by secreting some migration inhibitory factor. Accordingly, we cultured leucocytes from infants and adults in capillary tubes and tested for the migration of leucocytes in the Kennedy chambers. There was complete inhibition of infant leucocyte migration with as little as 6 μg of protein equivalent from fetal cortical extract. Thus the leucocytes of infants may be presumed to be sensitized to the fetal cortical extract. What part it plays in the death of these cells and their involution we cannot say.

From our studies on human fetal adrenal cortex, we have to explain why does not a fetus of earlier gestation period recognize this component as foreign or else become tolerant to it. Our explanation is, as long as the placenta secretes enough estrogens and gonadotrophins, the hormones, probably have a high affinity for this fetal adrenal component and prevents recognition of the fetal cortical cells.

The steroid hormones are known to have high affinity for certain proteins and thus may change the structure of repressor (which is most probably a protein) allowing the operon to proceed with the synthesis of mRNA [18] for let us say a vital protein for the fetal cortical cell. When the placental hormones become inadequate, no such binding can occur and, therefore, the operon becomes inoperative and this vital protein is no longer synthesized thus leading to the death of the fetal cortical cell.

It might also be that this fetal cortical component when combined with placental hormones cannot be recognized as foreign cells by the immunologic apparatus of the fetus. When the placental hormones decrease in amount, there is no more binding, and the protein assumes a conformation which can be recognized as foreign component by the individual, who therefore, proceeds to deal with the foreign component by both humoral and cell mediated immune responses. The process is necessarily slow and takes place over a period of months instead of days. The fetal adrenal cells probably become subject to apoptosis and removed from the system.

It would have been ideal if we could have isolated the fetal component and studied its binding to the placental hormones. But it was not possible to do so.

Summary

1. The human fetal adrenal gland is characterized by a smaller peripheral rim of adultogenic cells and a much larger layer of what are termed fetal cortical cells. During the greater period of gestation the fetal cells, which occupy the central four fifths of the gland appear to dominate the morphology of the gland. Starting at the last couple of weeks before parturition the fetal cells go through a process of involution by which the whole fetal layer of cells dies out and by and by the adultogenic cells proliferate to form the ultimate mature adrenal gland.
2. The histology of the adrenal gland was studied from about 7 weeks of gestation to a few days after birth. The gland arises from a mesothelial condensation on each side. The two zones of the human fetal adrenal glands can be distinguished by the structure of the cells and also by staining capacity. Beginning at about 12–16 weeks, the medullary tissue which descends from migratory cells that leave the primitive ganglia of the celiac plexus of the autonomic nervous system, approach the capsule, probably split it, produce an indentation in the adultogenic layer, push it, and gradually become incorporated into the substance. Originally when they were outside the gland they probably were rounded masses, but when they travel inside the adrenal gland, they transform themselves into chords of cells. At the end of the gestation period the pretty polyhedral, preserved cells of fetal zones begin to look sickly, the cytoplasm degenerates and undergo dissolution and gradually disappear. But the adult zone proliferates to three zones i.e. zona glomerulosa, zona fasciculota, zona retucularis.
3. The soluble proteins of the fetal adrenal glands of different gestation period was studied by Polyacrylamide gel electrophoresis method and stained by amidosehwartz from 10 to 15 different kinds of protein could be identified in the extracts of the fetuses [19]; the pattern change from one period of gestation to another; some old proteins disappear, new proteins appeared, other increase in intensity and some decrease.
4. To find out the fetal specific protein which is present only in fetal gland but not in adult adrenal gland and vice versa. Rabbits were immunized against the buffered saline extract of fetal and adult adrenal gland and 1M NaCl extract of fetal and adult adrenal glands. The antigen antibody reactions were studied in ouchterlony system where antisera are given in central hole and specific and non specific antigen on the peripheral holes. When antibodies against saline extracts of fetal adrenal glands were reacted with saline extracts of fetal and adult adrenal glands, the reaction pattern showed that in addition to the common antigens, the adult saline extracts contained some antigens which were not present in the fetal saline extract. When antibodies against 1M NaCl extracts (membrane fraction) of the fetal adrenal gland was reacted with 1M NaCl extracts of fetal and adult adrenal glands, it was found that 1M NaCl extract of the fetal gland contained some proteins not present in the corresponding extract of the adult gland. When the fetal extract was treated with 1M NaCl extract of the adult gland, the adult components were precipited. Such treated rabbit antisera no longer reacted with 1M NaCl extract of adult adrenal glands but still reacted with 1M NaCl extract of fetal adrenal glands. The fetal glands, therefore, contained some specific components probably on their membranes which were not present in the adult adrenal gland.
5. By immunoprecipitation tests with infant serum, however, a higher quantity of protein was found to precipitate (serum dilution 1 in 10) with fetal adrenal extract than with adrenal extract of children, which did not contain any fetal component.
6. When sheep erythrocytes were either coated with tannic acid or trypsinized and then coated with saline extract of fetal adrenal gland, they were found to be agglutinated by infant's

serum up to a dilution of 1 in 20, but not by fetal serum. When such erythrocytes were coated with saline extracts of children adrenal glands no agglutination was found to occur with infant serum. The hemagllutination observed with infant's serum could not be detected with adult serum.

7. The infant in whom the involution of fetal adrenal cells is taking place, may manifest some cell mediated immune response to the fetal cortical cell, provided some immunological mechanism is at the basis of the involution. However, when both kinds of extracts of fetal and adult adrenal glands were injected intradermally into the forearm of 5, 6 month old infants, no induration could be detected at 2, 4, 24 and 48 h after the injection. No Arthus type of reaction was also demonstrable in as much as there was no discoloration at the site of intradermal injection when such infants were preinjected with Evan's blue (T 1824).
8. When leucocytes were prepared form the infant blood and cultured in the Kennedy chambers in the presence and absence of fetal cortical extracts in the medium, the fetal cortical extract was found to inhibit completely the migration of leucocytes from the capillaries.
9. The involution of the fetal cortical cell was, therefore, thought to be due to recognition of some fetal specific component as a sort of foreign proteins and consequent immunologic response of the infant. The failure of recognition during intrauterine life was presumed to result from altered configuration of the same protein, possibly by being attached to placental hormones.

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Development of the Human Fetal Brain

11

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Introduction

Growth is a very important feature of development of a multicellular organism. It involves both cell multiplication [1] and making more intra and extracellular material. Both types of increases involve the synthesis of DNA, RNA, Proteins, Lipids and other constituent materials of the cells. From the point of view of development, an important feature of growth is that it occurs to different extents in different parts of the developing embryo and different patterns of growth can continue for long periods [2].

The growth and maturation of an organ at the cellular level comprises four stages: (1) Proliferation, (2) Migration, (3) Differentiation and (4) Death. This sequence of events is observed in all parts of the brain; however, the timing differs from one cell type to another and from region to region and even within the brain there are remarkably complex differences in the rate of growth and differentiation between the various regions [3, 4]. This results in an extremely complex pattern of development when the brain is regarded as an organ.

The mechanisms involved in cell differentiation are almost certainly of a biochemical nature. Cells which have differentiated along a specific pathway are characterized among other things, by the spectrum of proteins they contain.

Proliferation

The formation of the brain, for example, in the rat, begins around the seventh gestational day, at about the time when implantation occurs in the uterus. Proliferation of cells occurs from the ventricular zone of the primitive brain regions around the 11th

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day and proceeds very rapidly [5] producing an enormous number of cells in a few days. Malnutrition during early pregnancy can reduce the number of cells formed, as measured by the DNA content of the brain [6, 7]. Human fetal nutritional requirements for normal growth are inferred either from comparisons between maternal diet during pregnancy and the size or condition of the baby at birth or from animal experiments [8, 9]. Protein (essential amino acid) or glucose deprivation results in an underdeveloped fetal brain [10]. It is generally assumed that a nutritionally excellent diet consumed by the mother during pregnancy is good for the fetus, while a poor diet will embarrass fetal nutrition and growth [11, 12]. Studies in Guatemala among rural American women of uniformly low socioeconomic state indicate that supplemental protein and energy intake during pregnancy are associated with increased birth weights and decreased morbidity and mortality among newborns [13].

Migration

After an unknown number of mitoses of stem cells, each newly formed cell of the central nervous system migrates out of the "proliferative compartment" [5] and form layers of homogeneous cells:

1. Different cell types separate from each other and cluster together.
2. Within the homogeneous cell population the cells align in parallel to each other. They show a basal-apical orientation.

The capacity of self-orientation is strictly limited to a short period during embryonic development.

Differentiation

During or following migration a second type of differentiation takes place: the formation of cell-specific structures. Neurons grow axons and dendrites and form synaptic interconnections. Glial cells differentiate into astroglia and oligodendroglia. The oligodendrocytes develop and differentiate their plasma membrane into myelin sheaths.

Cell formation and migration are accompanied by death of a certain number of newly formed cells [14]. Studied on the whole, the brain has shown that its rate of development is not linear but that at a certain moment suddenly increases dramatically. This period called "brain growth spurt period" [15] is characterized by the sudden formation of a great number of new cells (hyperplasic phase) which rapidly grow. The "growth spurt period" is associated with a rapidly increasing metabolic activity and the appearance of new functions. More detailed studies have shown that the time and duration of the "growth spurt period" differ not only from species to species [7, 16], but also from region to region and from cell type to cell type in the same species. This results in an extremely complex pattern of development for the whole brain of a given species.

Modification of hormone levels and of cellular environment appear to be important factors in CNS epigenesis. Malnutrition during development is one of the factors which appears to permanently impair CNS functioning particularly at the behavioral level and to provoke in humans permanent intellectual deficit.

The brain, the most complex organ of the body, takes long to mature. In some respects the brain is changing until the day we die; presumably every new memory that is formed depends on a modification of the functional organization of some part of the brain. However, the most rapid period of growth and anatomical change is during fetal and early postnatal life.

Dobbing and Sands [7] studied the growth and chemical development of the human brain. As in other parts of the body there is a fall in the percentage of water from about 92 % at 20–22 weeks gestation to 77 % in the adult. There is a simultaneous rise in the percentage of lipids in the form of myelin. Dobbing and Sands used cholesterol as an index of myelination since its accumulation bears a constant relation to cerebroside. There is a rapid rise in the concentration of cholesterol in the forebrain up to 4 years of age, followed by a small, gradual rise to adult values.

There are two main types of cells in the brain, those of the neurons and those of the glia. Dobbing and Sands were able to identify two phases of rapid cell multiplication in the forebrain. The first lasted from 10 to 18 weeks gestation and correspondent to the multiplication of neuroblasts; this process ceased at about 18 weeks gestation when the neuroblasts are differentiated

into neurons. At about this time glial multiplication began, and all further increase in number of cells was due to an increase in number of glial cells. In the later half of pregnancy there is rapid growth of the brain, of which one component is hyperplasia of the glial cells [17]. Over the same period the dendrites of the neurons which are laid down in the first half of pregnancy are also rapidly developing in size and complexity. The resulting change in brain weight is referred to as the brain growth spurt [9] and this continues well into the second postnatal year.

Growth in Numbers

The total number of cells in any organ can be measured by determining the total organ DNA content and dividing it by a constant for the particular species which represents the DNA content per diploid cell in that species [18]. Total brain DNA content represents the total number of brain cells and total cerebellar or cerebral DNA represents the total number of cells in each of those regions.

Once the number of cells is determined, the average weight per cell (total organ DNA: DNA content per cell) [19], protein content per cell can be determined simply by analyzing for each of these components and dividing by the number of cells. This can be expressed chemically as a weight/DNA, Protein/DNA ratio which may be a useful indication of cell size [20].

Anthropometric Measurement of the Human Fetal Brain

According to clinical studies done by our group at SSKM Hospital, Kolkata (mentioned in Chapter 6), from 1979 onwards on the measurement of growth and development of the human fetus including the fetal brain, there was a straight line relationship between the body weight and the weight of the brain (Fig. 11.1).

The weight of the whole brain up to the medulla oblongata formed 13.96–14.5 % of the body weight from 9 to 28 weeks of gestation (Table 11.1).

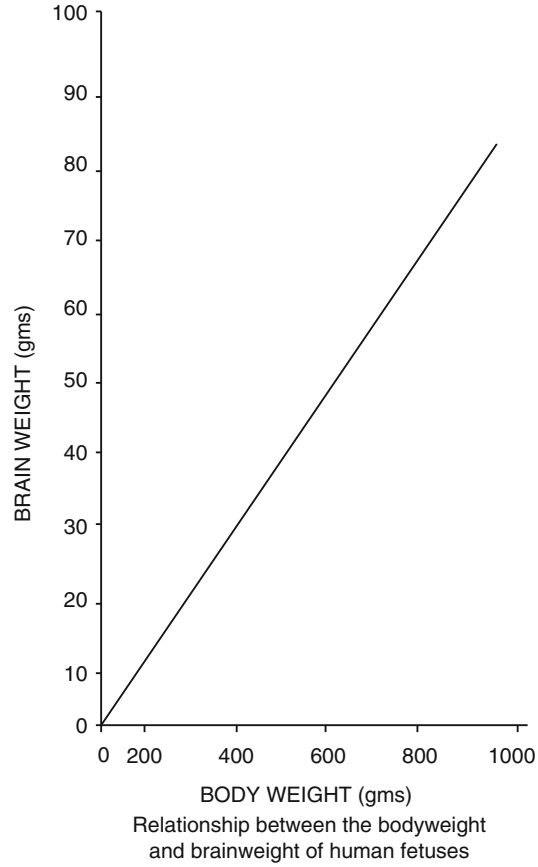

Fig. 11.1 Relationship between the bodyweight and brainweight of human fetuses

Table 11.1 Relationship between the body weight and the weight of brain of human fetus

Period of gestation (weeks)	No. of cases	Body weight (Mean ± S.D.) (gm)	Brain weight (Mean ± S.D.) (gm)	Brain weight per 100 g Body weight (gm)
9–12	74	8.73 ± 3.16	1.24 ± 0.50	14.45
13–16	208	48.94 ± 25.15	6.92 ± 3.59	14.14
17–20	192	186.62 ± 56.03	26.80 ± 8.15	14.24
21–24	68	419.88 ± 79.68	57.84 ± 13.10	13.96
25–28	27	737.48 ± 117.56	106.54 ± 20.00	14.47

Table 11.2 DNA content in developing human fetal cerebrum, cerebellum and midbrain

Period of gestation (weeks)	No. of cases	DNA content		
		Cerebellum (mg/g tissue)	Midbrain (mg/g tissue)	Cerebrum (mg/g tissue)
9–12	25	6.69 ± 0.26	3.55 ± 0.25	2.97 ± 0.27
13–16	38	5.51 ± 0.34*	2.85 ± 0.58***	2.38 ± 0.24*
17–20	46	3.49 ± 0.21**	2.25 ± 0.46***	1.54 ± 0.31*
21–24	23	2.69 ± 0.24***	1.44 ± 0.33***	1.33 ± 0.39***
25–28	01	2.01	1.30	1.22

Data are mean ± SEM
Statistics: P value, *P < .005; **P < .001; ***P > .05 (not significant), as compared with the preceding period of gestation

Table 11.3 Cell number of the developing human fetal cerebrum, cerebellum and midbrain

Period of gestation (weeks)	No. of cases	Cell number		
		Cerebrum	Cerebellum	Midbrain
9–12	25	$(1.24 \pm 0.37) \times 10^9$	$(0.10 \pm 0.03) \times 10^9$	$(0.24 \pm 0.08) \times 10^9$
13–16	38	$(5.28 \pm 1.97) \times 10^9$	$(0.23 \pm 0.09) \times 10^9$	$(0.41 \pm 0.21) \times 10^9$
17–20	46	$(16.95 \pm 5.63) \times 10^9$	$(0.45 \pm 0.12) \times 10^9$	$(0.48 \pm 0.15) \times 10^9$
21–24	23	$(27.64 \pm 3.44) \times 10^9$	$(0.87 \pm 0.28) \times 10^9$	$(1.28 \pm 0.37) \times 10^9$
25–28	01	31.96×10^9	1.42×10^9	2.08×10^9

Data are mean ± S.D.

Biochemical Changes in the Human Fetal Brain

DNA and RNA Content

The brain was divided into three regions, (A) The two cerebral hemispheres, (B) The cerebellum and (C) The mid and hind brain up to the lower end of the medulla oblongata at the level of the first cervical vertebra. In the course of gestation there was a progressive decrease in the DNA content per gm of the nervous tissues from 9 to 28 weeks of gestation. In the cerebrum the rate of decrease was more or less uniform which is shown in the Table 11.2. In the cerebrum and midbrain the rate of decrease was more or less the same up to 24 weeks; but the rate of decrease slowed down to 10 % from 24 to 28 weeks.

The total number of cells in the brain was calculated from the DNA content and the total weight, taking 6.2 pg as the DNA content per cell which is shown in Table 11.3. There was about 30-fold increase in the number of cells from 9 to 28 weeks of gestation in the cerebrum, 15-fold in the cerebellum and eightfold in the midbrain.

The total number of cells per mg of tissue was higher at earlier weeks of gestation in all areas of the brain. Thus at 9–12 weeks of gestation the number of cells per mg of the cerebral tissue was about 10^9 and at 13–16 weeks 0.8×10^9, at 17–20 week 0.5×10^9. It is apparent, therefore, that during growth and differentiation there was accumulations of cellular and extracellular materials. There was a decrease of RNA per mg of tissue form 9–24 weeks in the cerebellum and midbrain than in the cerebrum which is shown in Table 11.4.

The increase in the weight of the tissue per unit area is, therefore, not due to accumulation of RNA, grossly speaking. There might, however, be a different micro-environmental change not detectable by total amount of RNA during the progress of gestation.

Protein Content

Total protein content of the human fetal brain at various period of gestation was studied. The protein content in mg per gm of wet weight (Table 11.5a) and the protein content in mg per gm dry weight (Table 11.5b) of cerebrum, cerebellum and midbrain decreased significantly from 13 to 16 weeks of gestation. It was also noted that the

Table 11.4 RNA content in developing human fetal cerebrum, cerebellum and midbrain

Period of gestation (weeks)	No. of cases	RNA content		
		Cerebrum mg/g tissue	Cerebellum mg/g tissue	Midbrain mg/g tissue
9–12	25	3.243 ± 0.27	1.825 ± 0.28	1.579 ± 0.19
13–16	38	3.178* ± 0.20	1.650* ± 0.23	1.380** ± 0.09
17–20	46	2.316*** ± 0.32	1.360** ± 0.07	1.060*** ± 0.11
21–24	23	2.073* ± 0.21	1.66*** ± 0.11	0.980* ± 0.09
25–28	01	1.98	1.41	1.30

Data are mean ± SEM
Statistics: P value, *P > 0.05; (not significant) **P < 0.005; ***P < 0.001, as compared with the preceding period of gestation

Table 11.5a Protein content in human fetal cerebrum, cerebellum and midbrain

Period of gestation (weeks)	No. of cases	Protein content		
		Cerebrum mg/g wet tissue	Cerebellum mg/g wet tissue	Midbrain mg/g wet tissue
9–12	11	63.81 ± 1.98	61.90 ± 0.95	60.13 ± 1.71
13–16	19	60.70 ± 1.10**	59.21 ± 0.68*	58.89 ± 0.55*
17–20	16	58.11 ± 1.66**	57.30 ± 0.72*	56.91 ± 0.26*
21–24	9	57.50 ± 2.03*	56.42 ± 0.79*	55.01 ± 0.95**

Data are mean ± SEM

Table 11.5b Protein content in human fetal cerebrum, cerebellum and midbrain

Period of gestation (weeks)	No. of cases	Protein content		
		Cerebrum mg/g dry tissue	Cerebellum mg/g dry tissue	Midbrain mg/g dry tissue
9–12	11	709 ± 32.7	687 ± 19.9	668 ± 38.5
13–16	19	607 ± 30.6*	592 ± 14.7*	589 ± 13.8*
17–20	16	581 ± 25.2****	573 ± 10.6*	569 ± 10.9*
21–24	9	575 ± 25.9***	564 ± 6.8	550 ± 18.9**

Data are mean ± SEM
Statistics: P value, *P < 0.001; **P < 0.005; ***P > 0.05, **** < 0.01; (not significant) as compared with the preceding period of gestation

protein content of the above regions were nearly the same at the corresponding period of gestation.

^{14}C-leucine incorporation was studied in the human fetal brain at different gestational periods. It was found in Table 11.6, that 16,000 g with supernatant incorporation in the cerebrum and midbrain the rate of incorporation increased from 13 to 20 week of gestation and then declined up to 24 weeks of gestation to values even lower than those at 13–16 weeks of gestation in the respective tissues.

Incorporation of ^{14}C-leucine into proteins of mitochondrial fractions (16,000 × g pellet) of human fetal brain tissue appeared to be highest in cerebrum followed by midbrain at earlier periods of gestation (13–20 week). The rate of incorporation into the cerebrum protein remained almost unchanged throughout the period of gestation studied. The incorporation rate in the midbrain increased gradually with progress of gestation. Here also a twofold increase was found at 21–24 weeks of gestation.

P^{32} Incorporation in DNA, RNA and Phospholipid

We studied P^{32} incorporation into phospholipid fraction (Table 11.7) and in Acid soluble fraction, Nucleotide, DNA and RNA (Table 11.8). We also studied P^{32} incorporation into total Nucleic Acid (Table 11.9). All the studies were done in fetal

Table 11.6 In vitro incorporation of L-(^{14}C-U)-leucine into TCA precipitable proteins from 16,000 × *g* supernatant and of pellet of human fetal brain tissues

Period of gestation (weeks)	No. of cases	Incorporation (c.p.m/mg protein/10^4 counts)			
		Supernatant		Pellet	
		Cerebrum 120 min	Midbrain 120 min	Cerebrum 120 min	Midbrain 120 min
13–16	4	5.06 ± 0.24	3.12 ± 0.32	5.04 ± 0.35	1.53 ± 0.26
17–20	5	6.25^b ± 0.41	3.42^d ± 0.24	5.17^c ± 0.27	1.95^c ± 0.22
21–24	5	4.72^b ± 0.44	3.01^d ± 0.16	5.72^c ± 0.38	2.84^b ± 0.20

Each value represents the Mean ± SEM for 4–5 determinations
$^a{}_b$ < 0.001; $^b{}_p$ < 0.005; $^c{}_p$ < 0.01 and $^c{}_p$ < 0.05; $^d{}_p$ < 0.05 (not significant), compared with the preceding period of gestation

Table 11.7 P^{32} incorporation into lipid fractions of different human fetal brain

Period of gestation (weeks)	No. of cases	Incorporation (c.p.m/g tissue/100 counts)		
		Cerebrum	Cerebellum	Midbrain
9–12	8	10.05 ± 2.32	N.D.	22.11 ± 2.71
13–16	10	18.12 ± 2.83	19.10 ± 2.05	15.50 ± 3.15
17–20	7	14.51 ± 3.05	9.42 ± 2.91	13.69 ± 2.85
21–24	6	13.33 ± 2.19	5.91 ± 2.75	12.17 ± 3.06
Adult	2	N.D.	N.D.	N.D.

The results are expressed as mean ± S.D.
N.D. not done

Table 11.8 P^{32} incorporation into nucleic acids of different human fetal organs at different period of gestation

Period of gestation (weeks)	EE	Incorporation (c.p.m/g tissue/100 counts incubated $\times 10^{-2}$)		
		Cerebrum	Cerebellum	Midbrain
13–16	10	10.5 ± 2.19	8.83 ± 2.10	6.52 ± 1.84
17–20	7	5.79 ± 1.84	3.69 ± 1.13	3.10 ± 1.66
21–24	6	4.91 ± 1.06	3.60 ± 1.56	2.90 ± 1.07
Adult	2	N.D.	N.D.	N.D.

The results are expressed as mean ± S.D.
N.D. not done

Table 11.9 P-incorporation into acid soluble nucleotides of different human fetal organs at different period of gestation

Period of gestation (weeks)	No. of cases	Incorporation (c.p.m/g tissue/100 counts incubated)		
		Cerebrum	Cerebellum	Midbrain
9–12	8	5.31 ± 1.82	N.D	6.51 ± 1.51
13–16	10	4.32 ± 1.30	2.10 ± 0.4	5.13 ± 1.80
17–20	7	4.57 ± 1.20	3.52 ± 1.0	4.79 ± 1.03
21–24	6	5.98 ± 2.15	3.81 ± 1.31	6.83 ± 1.62
Adult	2	N.D.	N.D.	N.D.

The results are expressed as mean ± S.D.
N.D. not done

Cerebrum, Cerebellum and Midbrain of human fetuses at different periods of gestation. The results showed that incorporation of P^{32} into total Nucleic acid was less than into the lipid fraction. P^{32} incorporation into nucleic acid were highest in the earlier period of gestation, i.e., 13–16 weeks, than in the later periods.

Synthesis in the Human

Glutamine synthetase catalyzes a number of reactions [21], among which there are two important reactions. The first reaction catalyzes glutamine formation from glutamate, ammonia and ATP and the second causes the formation of

gamma-glutamyl hydroxamate from glutamate, hydroxyl amine and ATP. No detectable amount of glutamine synthetase activity was found in the human fetal brain using the first reaction. The enzyme activities of the human fetal cerebrum, cerebellum and midbrain were studied. In all the fetal organs that were studied, namely cerebrum, cerebellum and midbrain, the enzyme activity of the second reaction showed two to threefold increase from 13 to 28 weeks of gestation. The increase was progressive throughout the period of gestation.

Histological Studies of the Human Fetal Central Nervous System

Only some aspects of the histology of the central nervous system in developing human fetuses are illustrated. Figure 11.2 shows the disposition of the neural tube in a human fetus of below 7 weeks of gestation at a very low magnification. The total field of photomicrograph was obtained by joining the photomicrographs of five different parts carefully. The neuroblasts arise from the ependyma of the neural canal. Even at this stage there is some differentiation of the neuroblasts pushing towards the periphery (Fig. 11.2). The ventricular zone is a densely stained layer of cells with evidences of a layer of cells migrating towards the periphery. The intermediate zone contains fibres and sparsely situated cells, while the marginal layer contains fibres only. There is a layer of nerve fibres surrounding the neural tube (Fig. 11.3). The fetus has 22–24 somites. At some places the growing neurons seem to innervate growing muscular tissues within the somite (Fig. 11.4). The transition between the deeply staining packed neuroblasts and the differentiating sparsely disposed neural cells is rather abrupt (Fig. 11.7). Figure 11.8 shows the general appearance of the central nervous system at the cranial end. The cells are less differentiated than the cells at the level of the spinal canal (Figs. 11.2, 11.3, 11.4, 11.5, 11.6, 11.7, and 11.8). The ventricles are lined by almost a single layer of epithelial cells from which (perhaps?) the generally undifferentiated cells arise and spread (Fig. 11.9). Incidentally, the picture shows a beautiful reproduction of the developing eye (Figs. 11.10, 11.11, and 11.12). Figure 11.13 shows the development around a capillary with nucleated cells (1.725 g fetus) and Fig. 11.14 shows the histology of a part of the cerebrum of a fetus weighing 2.3 g. It illustrates the rapid growth of the invaginations of the cephalic ends of the neural

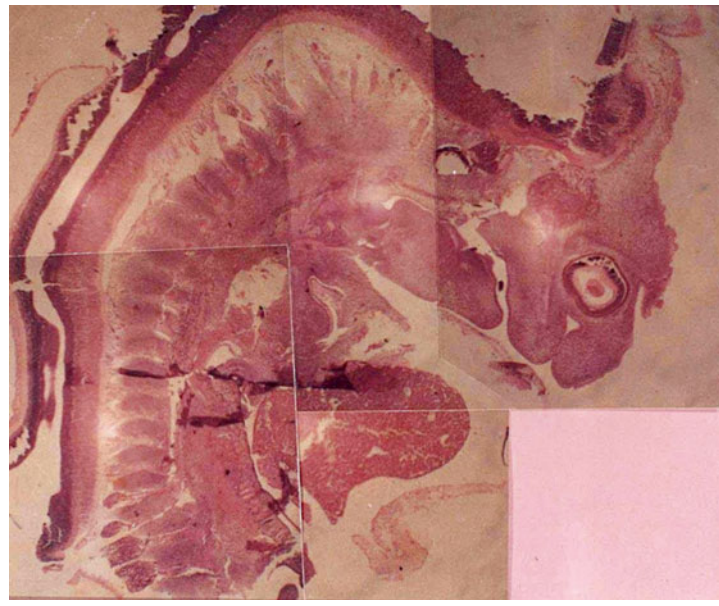

Fig. 11.2 Photomicrograph of a sagital section in a human fetus of below 7 weeks of gestation showing the disposition of the neural tube at a very low magnification

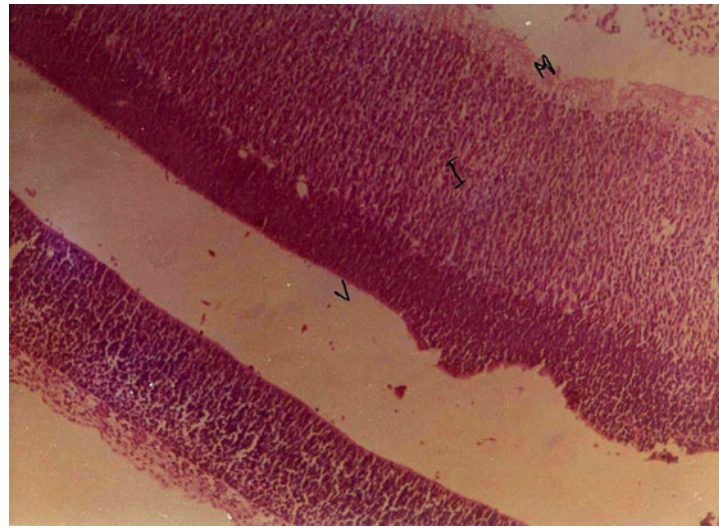

Fig. 11.3 High power field of neuroblasts showing the ventricular zone, intermediate zone and the marginal layer of the human fetus below 7 weeks of gestation

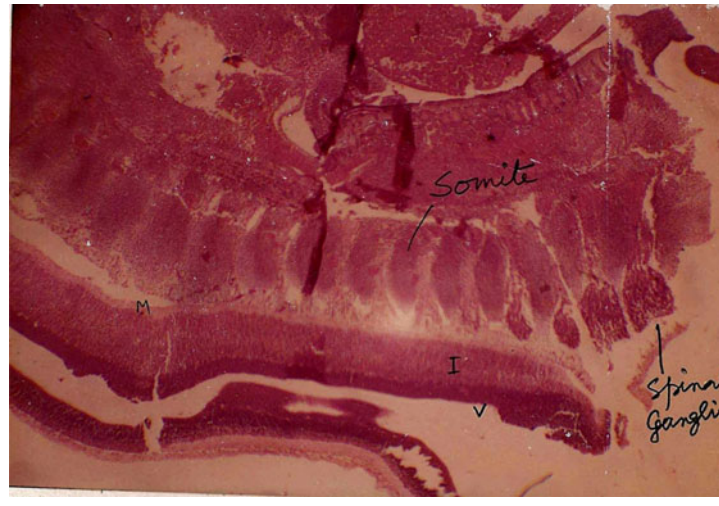

Fig. 11.4 Layer of nerve fibers surrounding the neural tube

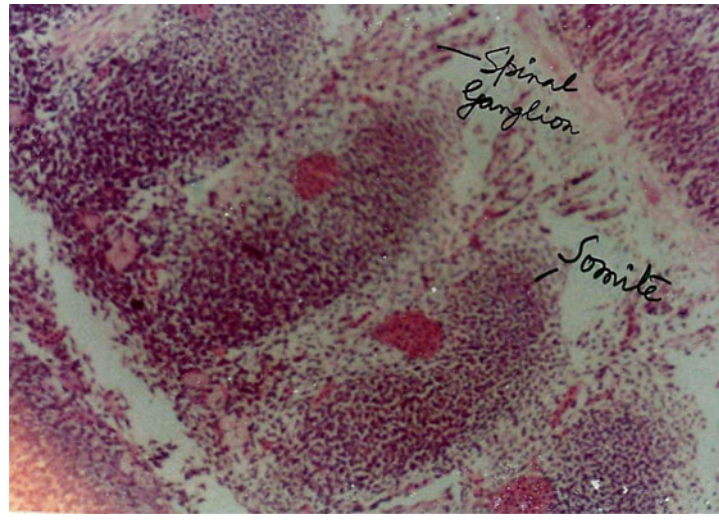

Fig. 11.5 Somites with innervations growing neurons to muscular tissue

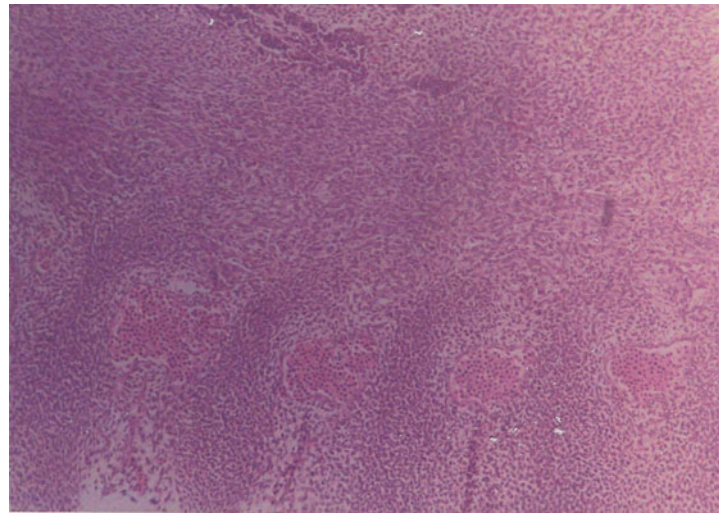

Fig. 11.6 Somites containing centrally located blood vessels

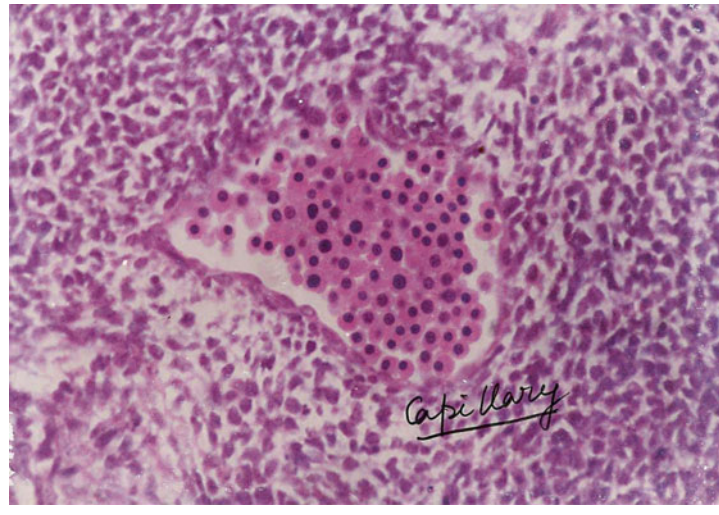

Fig. 11.7 High power field of a blood vessel containing nucleated erythrocytes within the somites

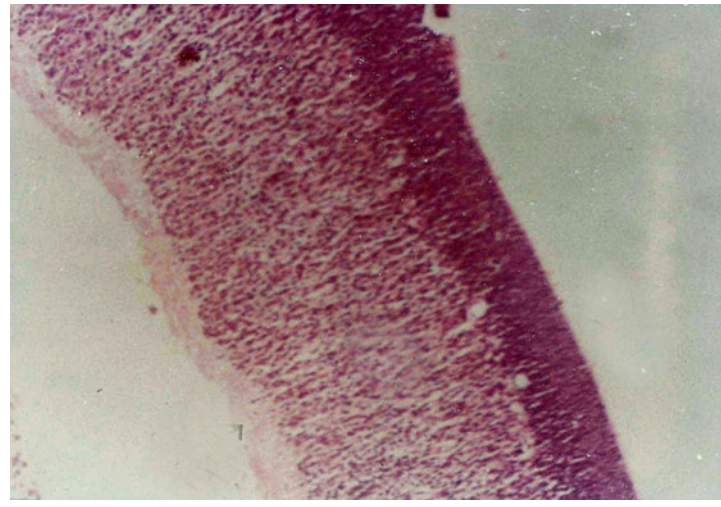

Fig. 11.8 High power field of neuroblasts

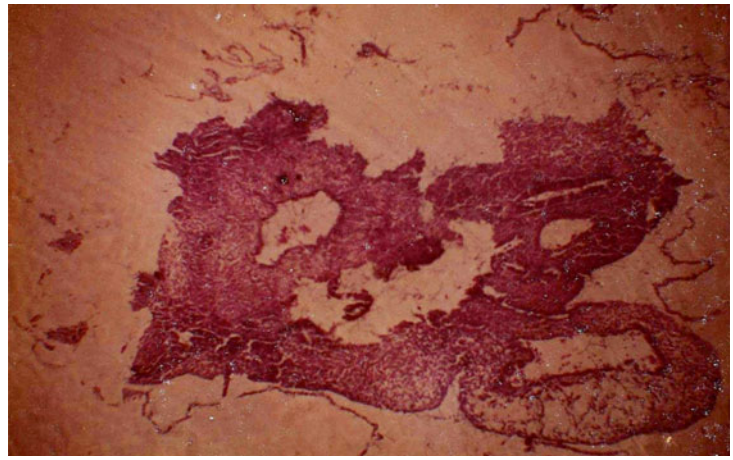

Fig. 11.9 Low power view of CNS at the cranial end of 100 mg fetus

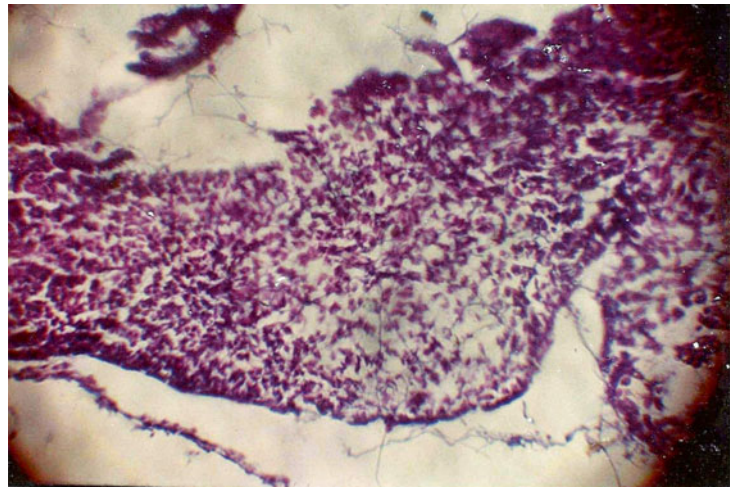

Fig. 11.10 High power view of the CNS of 100 mg fetus showing relatively uniform looking undifferentiated cells

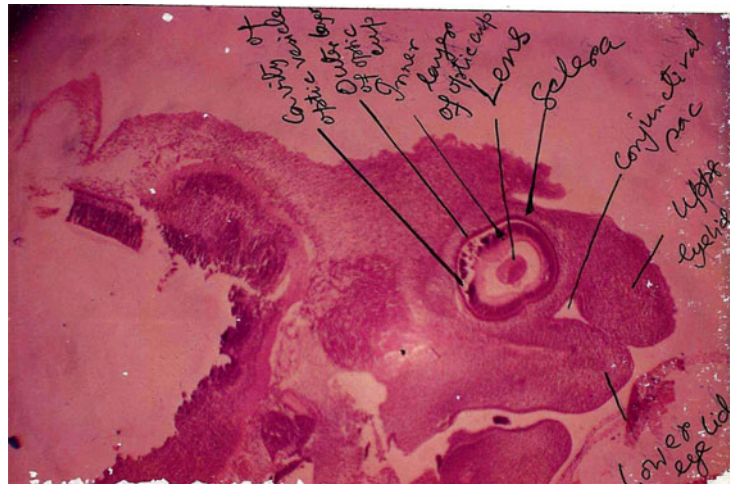

Fig. 11.11 Low power view of a developing eye (below 7 weeks)

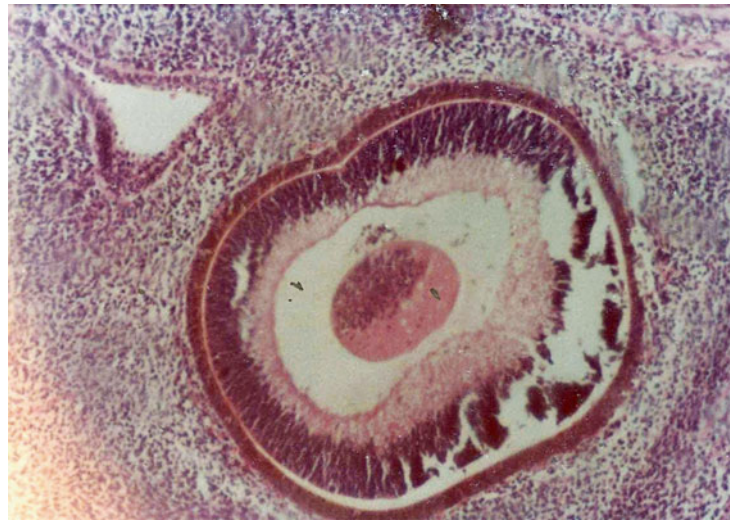

Fig. 11.12 High power view of developing eye (below 7 weeks)

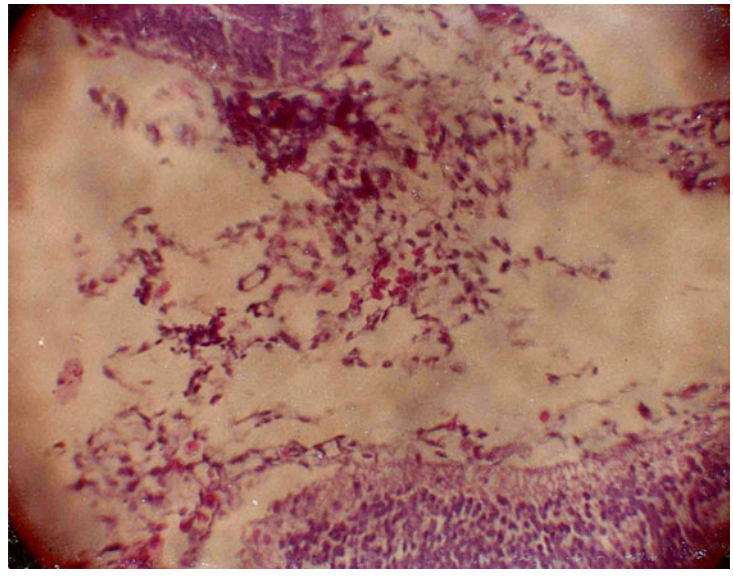

Fig. 11.13 High power field showing development around a capillary with nucleated cells (1.725 g fetus) at the upper part

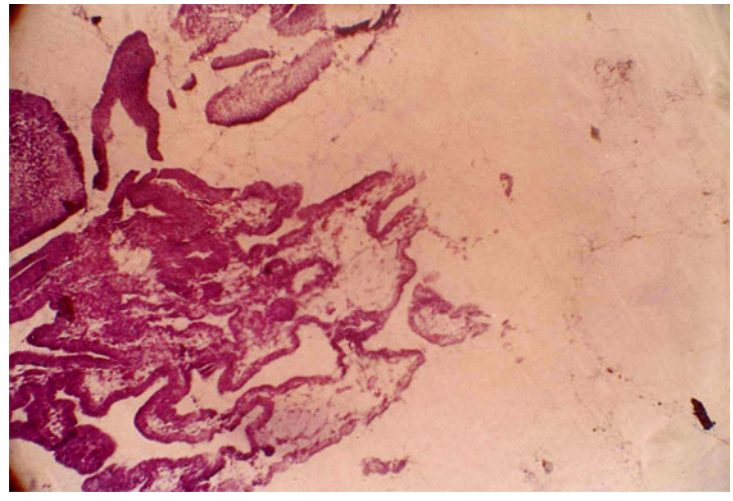

Fig. 11.14 Low power view of cerebrum of a 2.3 g fetus showing development of layers

tube with mostly undifferentiated neuroblasts, whereas, in other places (Fig. 11.15) the same brain is consolidating the neuronal growth to be differentiated further, later on, in life. Figure 11.16 shows the histology of the part of the cerebrum of fetus weighing 36 g (about 14 weeks of gestation). It shows extensive growth of capillaries within the developing neuronal cells. Figure 11.17 shows the histology of the cerebrum of a fetus weighing 95.5 g (about 16 weeks of gestation).

It shows the development of a sulcus in the brain. At about this time there is extensive capillary growth within the developing cerebrum. Figure 11.18 gives a view of the cerebrum-subgerminal layer showing at least five capillaries (167 g fetus). Figure 11.19 gives a view of the sub-cortical region showing fibers and a capillary (224 g fetus), and Figure 11.20 gives a view of an area of the midbrain of a 12 g fetus showing peripherally the cells circumscribed by fibrous layer (perhaps tract) and smaller islands of neurons. Figure 11.21 shows the histology of cerebellum of a fetus weighing 90 g (about 16 weeks of gestation). The external granular layer is in the proliferative stage. Figure 11.22 shows the histological appearance of cerebellum of a fetus weighing 150 g (about 17 weeks of gestation). There are only two layers, an outer granular and an inner molecular.

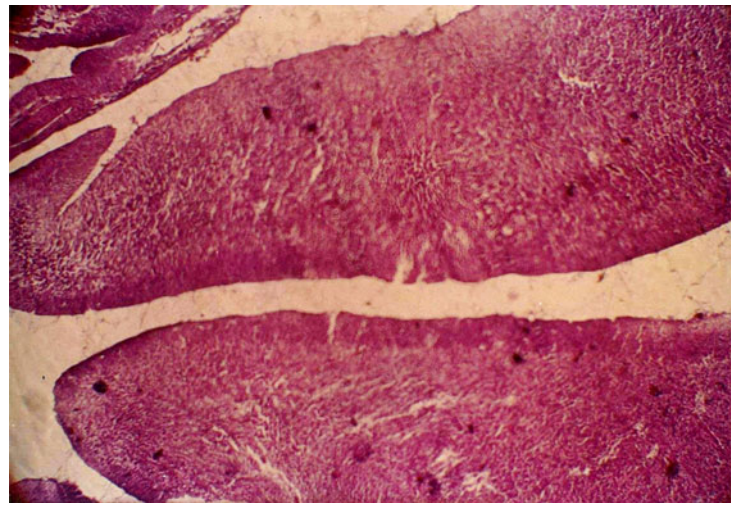

Fig. 11.15 Low power view of cerebrum of 2.3 g fetus in another place showing the neuronal migration

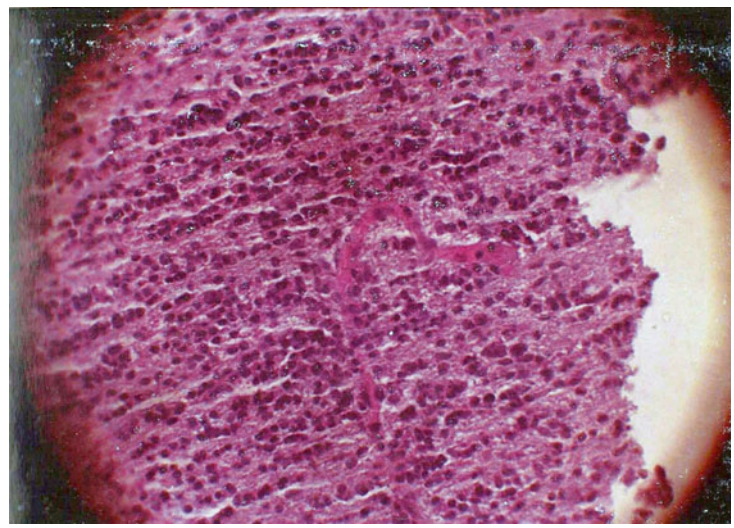

Fig. 11.16 High power view of extensive capillary network in the cerebrum (36 g fetus)

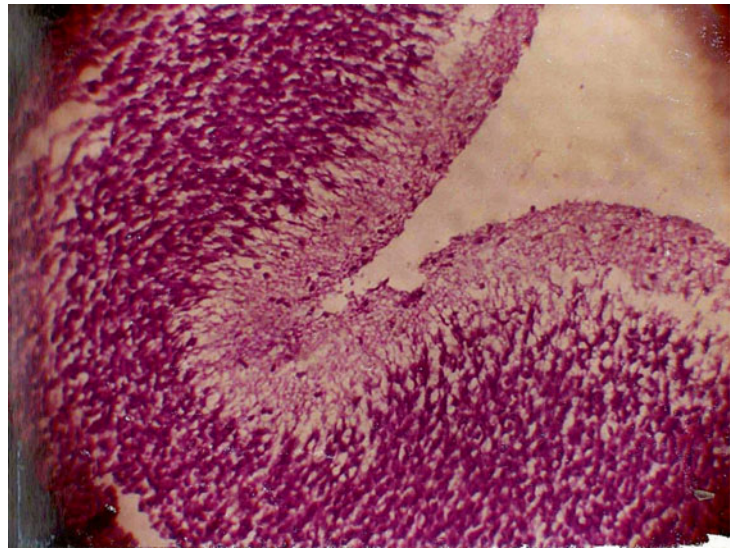

Fig. 11.17 High power view of cerebrum showing development of sulcus (95.5 g of fetus)

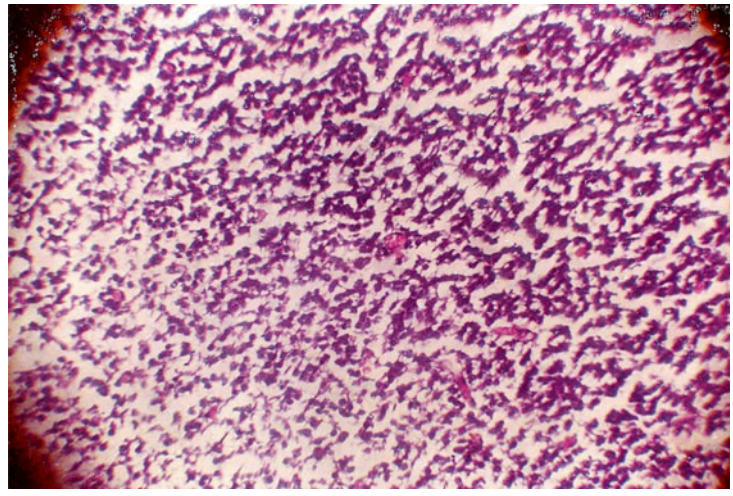

Fig. 11.18 High power view of cerebrum-subgerminal layer showing at least five capillaries (167 g fetus)

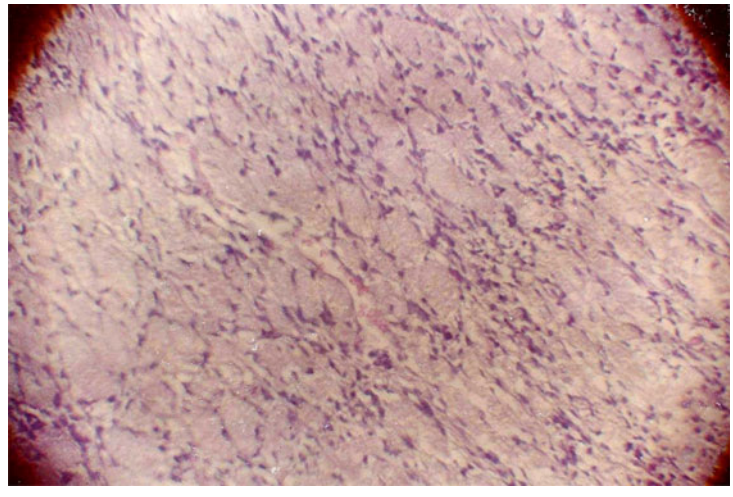

Fig. 11.19 High power view of sub-cortical region showing fibers and a capillary (224 g fetus)

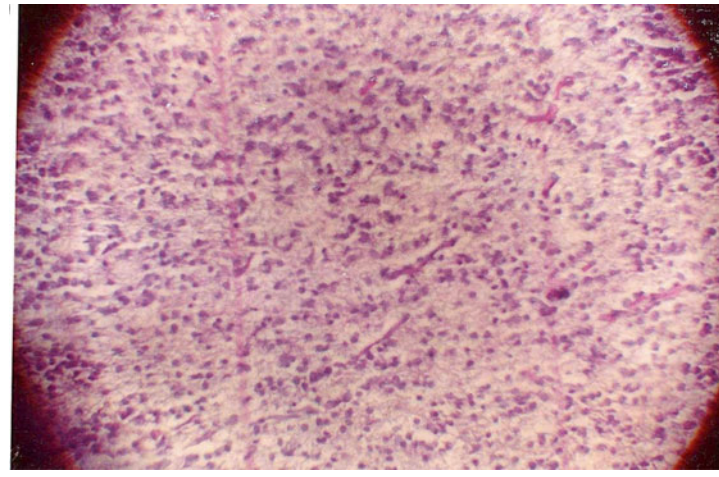

Fig. 11.20 High power view of an area of the midbrain of a 12 g fetus showing peripherally the cells circumscribed by fibrous layer (perhaps tract) and smaller islands of neurons

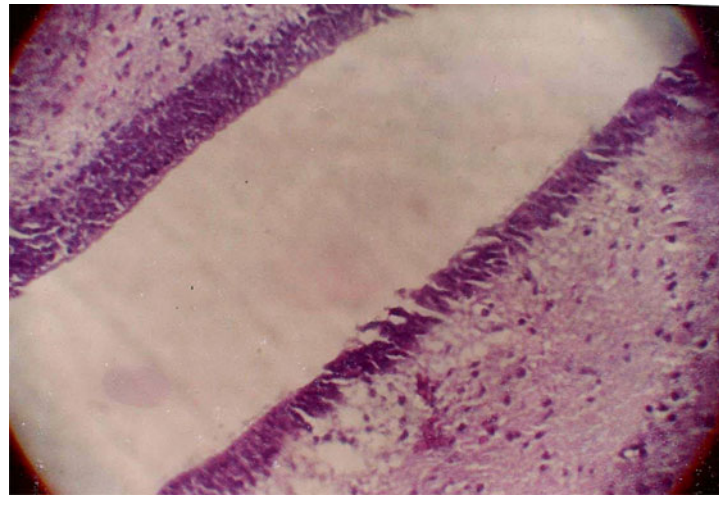

Fig. 11.21 High power view of mid brain of 90 g of fetus showing peripheral layer of neuronal cells and central layer of more fibers

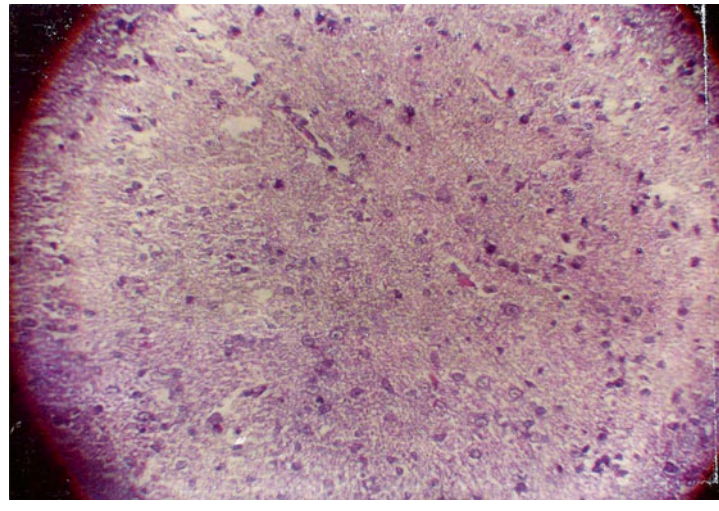

Fig. 11.22 High power view of mid brain of 150 g fetus showing two capillaries in the central part of the fiber rich area

There is no evidence of the single layer of the purkinje cell.

Discussion

Anthropometric Measurement

The rate of growth of a particular human fetus depends upon its intrinsic growth potential, inherent in its genomic complex which is modified by the environment, provided locally by the mother's uterine complex. Individual growth of the fetus is a function of the genetic potential.

Nucleic Acid Contents

DNA contents per gram wet weight of human fetal cerebrum, cerebellum and midbrain decreased with progress of gestation. Such a decrease in the brain was also described by Subba Rao and Janardana Sarma [22]. This can be explained by the fact that the growth of the brain entails not only of cellular growth but of the neuronal processes as well. In the earlier periods of gestation the cellular element was more but as more and more processes develop in the course of differentiation, cells per unit mass of the brain naturally will decrease. It was noted that the rate of cell multiplication was more in the earlier period of gestation. However, this rate varies in different parts of the brain. For example, in the midbrain which consists predominatly of white matter, the rate of increase is slow. The rate of growth in the cerebellum does not decrease with progress of gestation as much as in the cerebrum. This has also been the experience of other workers, although Winick et al. [23] think that the rate of cell growth in the cerebrum and cerebellum is similar.

Enzymes: Glutamine Synthetase

Glutamine Synthetase catalyzes not only synthesis of glutamine from glutamate, NH_3 and ATP, but also has three other enzymatic activities one of which is formation of gammaglutamyl hydroxamate and ammonia from glutamine and hydroxyl amine. In human fetal organs, including liver, cerebrum, midbrain and cerebellum synthesis of glutamine could not be detected from glutamate, ammonia and ATP.

Nor was it possible to detect any activity of forming gamma-glutamyl hydroxamate and ADP from glutamate, NH_2OH and ATP. The third reaction, i.e., formation of gamma-glutamyl hydroxamate and NH_3 from glutamine and NH_2OH could be demonstrated in these human fetal organs.

Glutamine synthesis has been speculated to be a means of detoxication of ammonia locally produced [24–26]. Most of the ammonia, in this process, comes from the dehydrogenation of glutamic acid. Glutamic acid is an alternate metabolic fuel for the brain, apart from glucose and ketone bodies. The glutamine produced in the brain may diffuse into the plasma and serve as the building blocks of protein, and also as a means of bicarbonate formation in the kidney tubules in conditions of acidosis.

In Vitro ^{32}P: Incorporation

Incorporation of ^{32}P into acid soluble nucleotides of liver decreases in course of gestation. The higher incorporation at earlier periods of gestation indicates faster metabolic turnover in general. The nucleotides are utilized for biosynthesis of various organic compounds. At earlier periods of gestation cell division takes precedence over other metabolic activities. Thus the neucleoside triphosphates are more utilized for the biosynthesis of the nucleic acids. In our own experiments the total DNA contents increased about six times from a gestation period of 9–12 to 13–16 weeks, whereas they increased less than two times only from a gestation period of 21–24 to 25–28 weeks. In later periods of gestation, other metabolic activities involving biosynthesis, which all require nucleoside triphospahte, increase.

However the rate of cell division decreases thus accounting for the overall decrease of formation of acid soluble nucleotides in fetal livers of later gestation periods. However, it is not possible to account for similar rates of incorporation of ^{32}P into acid soluble nucleotides of the brain. The total

Table 11.10 Gamma-glutamyl transferase activity in human fetal brain

Period of gestation (weeks)	No. of cases	Specific Activity (μmol/h/g protein)		
		Cerebrum	Cerebellum	Midbrain
9–12	12	200.41 ± 30.32	N.D.	N.D.
13–16	33	708.43 ± 40.11**	761.33 ± 68.52	652.42 ± 46.15
17–20	27	870.91 ± 74.72*	1,204.73 ± 106.10**	1,300.81 ± 114.61**
21–24	18	911.35 ± 83.20***	1,422.54 ± 117.76****	1,853.22 ± 132.11**
25–28	07	1,312.46 ± 111.20**	1,508.97 ± 140.64***	2,018.16 ± 139.95****
	01	N.D.	N.D.	N.D.

Data are mean ± SEM
Statistics: P value, *P < 0.005; **P < 0.001; ***P < 0.01, ****P > 0.05; (not significant) as compared with the preceding period of gestation

DNA, for example, in the cerebrum increases about four times from 9–12 to 13–16 weeks of gestation (Table 11.10), whereas it increases only 1.5 times from 21–24 to 25–28 weeks. But the rate of incorporation of ^{32}P into acid soluble nucleotides is more or less the same in the above gestation periods. Perhaps in the case of brain, acid soluble nucleotides are very importantly and significantly involved in myelin synthesis thus overshadowing the effect of rate of synthesis of DNA.

In the fetal brain, there was again no gross difference in the ^{32}P incorporation into the lipids of the cerebrum and midbrain. In the cerebellum, however, the rate of incorporation diminished. The growth of the cerebellum differs in different species. In man there is a contention that the cerebellum continues to grow even after the cerebrum has stopped growing. Cell division is accompanied by membrane synthesis in the two daughter cells, which continues to grow after ceasation of mitosis. The issue is whether the decrease in incorporation of ^{32}P into phospholipids signifies rapid membrane growth.

In Vitro ^{14}C-Leucine Incorporation

During the growth of the human fetus in uterus, the brain increased in mass in proportion to the body weight similar to the liver, from 13 to 28 weeks of gestation. There are other human fetal organs like the adrenal cortex or femur which do not grow in proportion to the total body weight (our unpublished results). This difference in response to the growth potential may be related to the hormonal characteristics of the different organs, in terms of receptors for the various hormones, directly or indirectly related to growth, and also in terms of the pattern of response to the receptor bound hormones.

It may only be surmised that human fetal brain and liver from 13 to 28 weeks of gestation grow uniformly and unfold the receptors and respond to the receptor bound hormones in a uniform way. The protein content of the human fetal liver increased during progress of gestation. The water content decreased from 80% at 9 weeks to 77.5% at 24 weeks. The increase of protein content in the human fetal liver might have been due to increased rate of protein synthesis commensurate with assumption of specific functions in the economy of the developing fetus.

It may be stated that the slight reduction of protein content per unit dry weight of the tissue in brain regions of human fetuses is not due to reduction of protein synthesis during the period of gestation studied. Further, it may be suggested that the slight reduction of protein content per dry weight of the brain regions accompanied by an almost unaltered rate of protein synthesis is possibly due to the rapid accumulation of lipid components in the respective brain regions, indicating the comparatively high rate of lipid synthesis in the brain during the fetal life studied.

Incorporation of U ^{14}C- Glucose In Vitro

Radiolabelled glucose was incorporated into radioactive compounds in brain tissues in vitro. The incorporation into amino acids were

characteristic of adult brain tissues in contrast to the fetal brains. Very little incorporation of counts from glucose into either the lipid fractions or into nucleic acids could be demonstrated.

Histological Studies of the Human Fetal Central Nervous System

A detailed histological study of the human fetal central nervous system is beyond the purview of the present investigations. Only some points are being presented.

Development of the Eye

We have a histological study of a 35 mm human embryo. The photomicrograph shows that the inner and outer layer of optic cups seem to encircle the lens completely whereas conventionally the nervous layer is illustrated to fold back in front of the lens demarcating the anterior chamber and developing cornea. In the present study, the sclera and presumptive cornea form one complete sheet of tissue. We, therefore, present two possibilities:

1. The plane of the section of the fetus is responsible for the presented picture. But then there should have been at least traces of the folding back of the nervous tissues.
2. The development of the anterior chamber and cornea is a later phenomenon. However we have no evidence of the anterior chamber and of the cornea as yet. The cellular nature of the developing lens is well illustrated in the present histology of the eye. The small conjunctival sac is lined by epithelial cells.

The developing neural tube at this stage of fetal life contains a more or less uniform type of tissue, with a ventricular, subventricular, intermediate and marginal zone. This is true both in the spinal region as well as in the cephalic parts of the tube (Figs. 11.2 and 11.4). The neural tube is a closed system at this stage and the histology shows more differentiative neural tissues.

Development of the Somites

The other interesting histology is that of the somites. These paraxial mesodermal condensations are the forerunners of the dermotome, myotome and sclerotome. We can count about 25 somites in this particular fetus. At this stage the myocele has been obliterated by the proliferation of cells. The epitheloid cells have changed their character into mesenchymal tissue. The capillary has complete endothelial wall and within the capillary almost all cells are nucleated erythrocytes consistent with their (?) yolk sac origin rather than of hepatic origin. The picture is consistent with the existence of a nutrient vessel within each individual somite for its further development and differentiation. This description I could not find in any text book of human embryology.

Cortical Histogenesis

The earliest histology of the cerebral cortex is from a fetus of about 8 weeks' gestation. The capillaries contain nucleated erythrocytes. The cellular development is similar to that found everywhere in the central nervous system – neuroepithelial cells from the ventricles dividing and migrating to the pial surface. It is difficult to trace this migration by usual hematoxylin eosin staining. Except for a layer of cells lining the ventricles, which stain deeply, the rest of the cells form almost a continuum bridging the two layers of the neuroepithelium. The blood supply in the developing cerebrum is plentiful. The vessels dip into the cortical matter from the surface.

Development of the Midbrain

The general plan of histogenesis in the midbrain is basically similar to other areas of the brain. The blood vessels are numerous. A distinguishing feature are the centres of neuronal congregations deep within the white matter, which are perhaps in connection with specific centre of the midbrain.

Development of the Cerebellum

The histological development of the human cerebellum has been studied only in certain aspects. The development in fetal life has been well described by Uzman [27] and Langman [28]. The development in fetal life of human cerebellum consists of an external granular layer, an internal granular layer and a layer of ventricular neuroepithelial layer. The rapid proliferation of the external granular layer is well illustrated in Fig. 11.23. The zone consists of a broad band of cells more or less uniform in character, whose fibres from the white matter internal to the zones. The ventricular neuroepihelial cells migrate towards the surface, forming an internal granular layer, internal to the white matter formed by the proliferated external granular layer of cells (Figs. 11.24 and 11.25). Further development of the cerebellum occurs at periods of gestation later than 20 weeks and continues postnatally (Fig. 11.26).

Summary and Conclusions

1. There is a straight line relationship between the body weight and brain weight. Weight of the brain of the fetus is around 14 % of the body weight throughout the gestation period.
2. The total nucleic acid contents (DNA and RNA) in the human fetal brain increased with

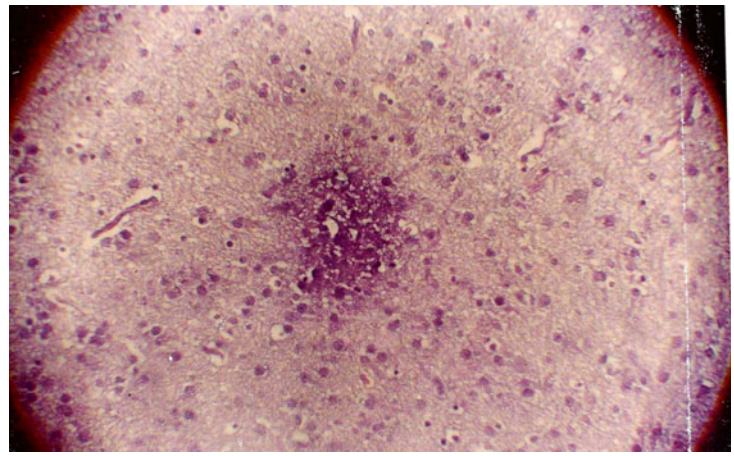

Fig. 11.23 High power view of mid brain of 347 g of fetus showing perhaps a cluster of neurons for either a cranial nerve centre or a group of neurons for a centre like the respiratory.(?)

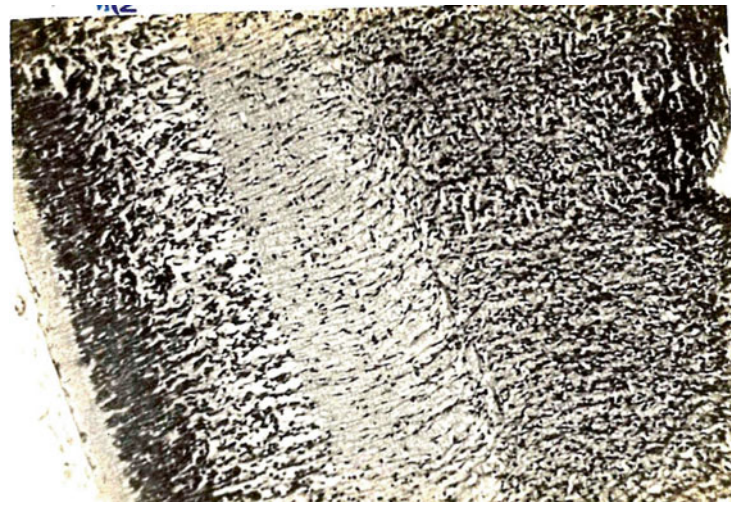

Fig. 11.24 Low power view of cerebellum of a 90 g fetus showing neuronal cells originating from two sides but distinctive characteristics in the two lines of origins

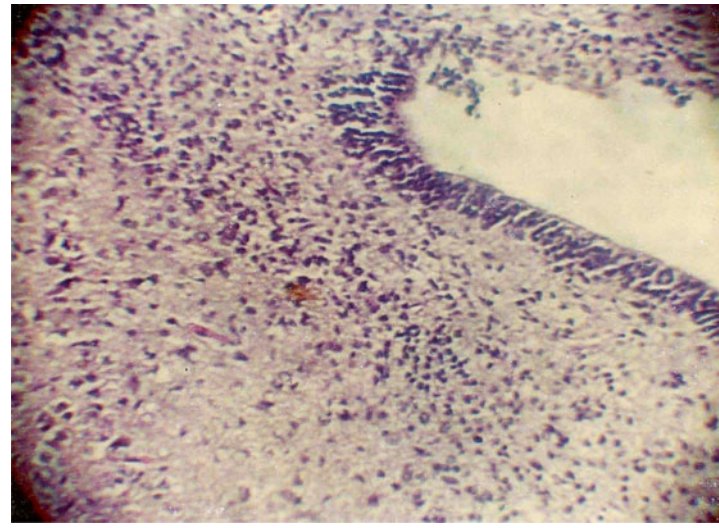

Fig. 11.25 High power view of cerebellum of a 150 g fetus showing cell migrating

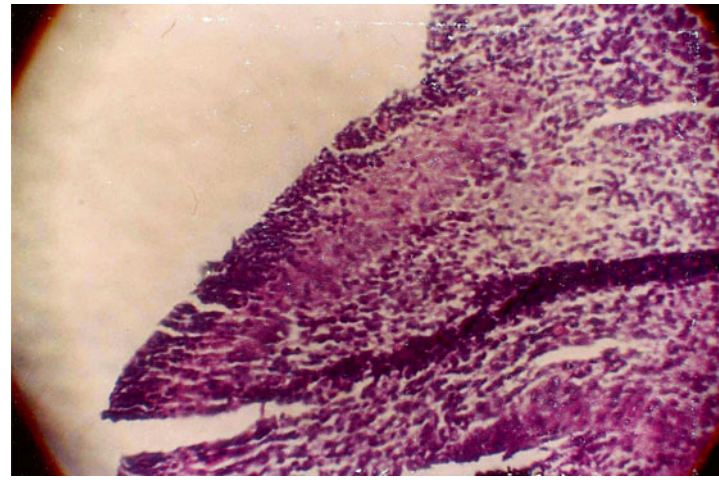

Fig. 11.26 Low power view of cerebellum of a 150 g fetus showing cell migration

the progress of gestation along with the increase of cell number of the organ. The rate of increase in total DNA content in early periods of gestation was much higher than at later periods. The DNA content per gm tissue decreased with progress of gestation. The rate of decrease in the cerebrum was more or less uniform while that in the cerebellum and midbrain was more or less the same up to 24 weeks and then further slowed down.

3. The number of cells increased 30-fold in the cerebrum, 15-fold in the cerebellum and eightfold in the midbrain form 9–12 weeks to 25–28 weeks of gestation. In the earlier stages of gestation, the growth of the brain entails cellular growth as well as neuronal processes. In earlier periods of gestation the cellular element was more but in the course of development due to more and more processes, cells per unit mass of the brain decreased.
4. The total protein content per gm wet wt of the human fetal brain was found to decrease with the increase of gestation.
5. No glutamine synthetase activity was detectable in the human fetal brain. Gammaglutamyl transferase activity increased progressively with gestation periods in the cerebrum,

cerebellum and midbrain. Glutamine synthesis is not a prominent feature of human fetal organs. The mammalian fetus is constantly supplied with glucose through the maternal circulation. So the alternate metabolic fuel, i.e., glutamic acid oxidation is perhaps not needed. A high level of glutaminase activity was found in the cerebrum and midbrain at 9–12 weeks of gestation. The neural tissues in earlier periods of gestation consist mostly of neurons rather than glial cells which may be relatively deficient in glutaminase activity.

6. In vitro incorporation of ^{32}P into the acid soluble nucleotides of human fetal liver slices decreased with progress of gestation. The rate of incorporation of ^{32}P into acid soluble nucleotides in the brain was more or less the same throughout the gestation studied. This signifies that perhaps in the case of the brain, the acid soluble nucleotides are very importantly and significantly involved in myelin synthesis. ^{32}P incorporation into lipids was much less than in acid soluble nucleotides. More incorporation in the earlier periods of gestation than in the later periods might signify higher metabolic activity and turnover at earlier periods.
7. The rate of ^{14}C- leucine incorporation into protein gradually increased up to 20 weeks and then declined. The tissue in brain regions of human fetuses at the later gestation periods accompanied by an almost unaltered rate of protein synthesis is possibly due to rapid accumulation of lipid components indicating a comparative high rate of lipid synthesis in the brain during the fetal life studied.
8. The amount of incorporation of ^{14}C- glutamate was more than that of ^{14}C- leucine at each gestation period. This suggests that glutamate has probably a special role to play in brain metabolism.
9. The incorporation of U-^{14}C-glucose into human fetal brain slices suggested that the fetal brain can utilize glucose for energy metabolism.
10. From the seventh week to the 28th week, the total weight of the brain forms almost a constant percent of the body weight. Certain aspects of the development of the eye were was included in the histological studies. The histological picture closely followed the pattern described by standard text books of embryology except in certain minor points. During early fetal life each somite has a centrally located blood vessel which supplies presumably nutrients for the further development of the somites. The histological development of the cerebrum, cerebellum and midbrain followed closely the description in standard text books of embryology.

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Lipid Metabolism in the Human Fetus Development

12

Ornella Guardamagna and Paola Cagliero

Abbreviations

ABCA1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
ApoA-1	Apoprotein A-1
ApoA-4	Apoprotein A-4
ApoE	Apoprotein E
CE	Cholesteryl ester
CETP	Colesteryl ester transfer protein
CHO	Cholesterol
HDL	High density lipoprotein
FA	Fatty acid
FFA	Free fatty acid
LCAT	Lecithin:cholesterol acyl transferase
LDL	Low density lipoprotein
LP	Lipoprotein
LRP-1	LDL-receptor related protein 1
LRP-2	LDL-receptor related protein 2
NPC1L1	Niemann-Pick C1-like1
PTP	Phospholipid transfer protein
SPC-X/2	Sterol carrier protein X and 2
SR-B1	Scavenger receptor class B
TG	Triglyceride
VLDL	Very low density lipoprotein

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Introduction

Lipids including cholesterol (CHO) and fatty acids (FAs) are main constituents of human body cells and actors in physiological functions thus representing a critical requirement for the embryonic and fetal development.

CHO and FAs attain multiples functions: CHO is a cellular membrane constituent, a steroid hormone, bile acids and oxysterol precursor and it is essential for activation of various signalling pathway [1, 2]. CHO plays an important role before implantation, as a precursor of progesterone synthesis, and helps in maintaining the early pregnancy [3–7]. When the embryo is implanted in the uterine wall, CHO is determinant for the embryogenesis and morphogenesis and patterning of the central nervous system [8]. As well FAs and triglycerides (TGs) are cellular membrane constituents, represent an energy source and take part in neuronal and visual development [9].

The embrio and fetus do not come in direct contact with the maternal circulation thus are dependent upon tissues surrounding them to receive the nutritional support. These tissues are represented by the yolk sac and trophoblasts, early in the first trimester, then the placenta since the end of the first trimester and the second trimester [5]. The placenta is an hemochorial villous organ with multiple functions: oxygen and CO_2 exchange, nutrient absorbption and immune

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barrier. It represents a bridge connecting mother and fetus through the maternal-placental (uteroplacental) blood circulation and the fetal-placental (fetoplacental) blood circulation. The functional unit of the placenta is the chorionic villus which contains syncytiotrophoblast/cytotrophoblast, villous stroma and fetal vascular endothelium, layers that separate the maternal blood from the fetal circulation [10]. The yolk sac and the placenta provide an adequate nutrient supply by transporting a wide variety of maternal molecules to the embrio and fetus, including lipids, so promoting the intrauterine development. The transfer of some nutrients is regulated by the placenta itself through specific enzymes, receptors and transport proteins; others nutrients are directly metabolized by the placenta.

During gestation metabolic changes intervene with a shift from carbohydrates to lipids for maternal energy production in order to make nutrients available for the fetus [11]. Glucose is the main substrate that crosses the placenta but other factors may also contribute to the fetal growth. The fetus requires a substantial amount of lipids throughout its development, the lack of CHO affecting growth disorders [12]. To satisfy these needs maternal physiological hyperlipidemia is manifest in pregnancy; CHO, TGs and FAs concentrations increase in both maternal plasma and erythrocytes thus allowing the fetus to rapidly receive and store fat, which exceeds by far that of any other nutrient [13]. Maternal plasma CHO may increase through the 12th week of gestation while TGs reach the 150–300 % of increase in the third trimester of pregnancy [6, 14, 15]. The two lipoproteins (LPs) classes involved in supporting the placental CHO need are low density lipoprotein (LDL) and high density lipoprotein (HDL) [16–18]. A supply of CHO requirement as a precursor for the production of steroid hormones in the placenta is further critical [19]. Fetal steroid precursors of estrogens regulate the uptake of maternal LPs to promote the placental progesterone synthesis. Both estrogen and progesterone are thus key determinants in pregnancy maintenance and fetal growth so being evident the basic role of fetal and maternal LPs [20, 21] (Fig. 12.1).

Lipids Synthesis and Transport

The fetus has two potential sources of CHO and FAs that include the endogenous and exogenous metabolic supply. The endogenous pathway is represented by lipids synthesized by the embrio and fetus themself, the exogenous pathway concerns lipids provided by the maternal and placental circulation. Fetal CHO and FAs are thus either taken up from the maternal circulation or synthesized *"de novo"*.

In humans very low density LP (VLDL) carries CHO and TG from the liver where originates to peripheral cells, LDL carries mainly CHO. HDL represents the reverse CHO pathway deputed to carry out free CHO from

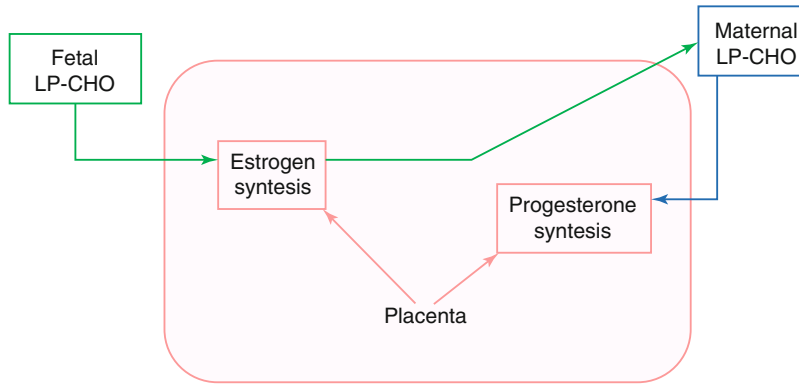

Fig. 12.1 Feto-maternal CHO intake and steroid hormonal synthesis regulation

peripheral cells, to promote CHO esterification to cholesteryl ester (CE), to provide exchange of CE and TG between circulating LPs and to bring up CE to the liver (Fig. 12.2). The delivery of lipids to the fetus is made available by the physiological maternal LPs increase which allow VLDL, LDL, HDL to be taken up by the placenta [6] (Fig. 12.3). Lipid metabolism undergoes particular changes during pregnancy, despite the fact that the placenta is practically impermeable to TGs, except for FAs. Through the early two third of pregnancy the mother

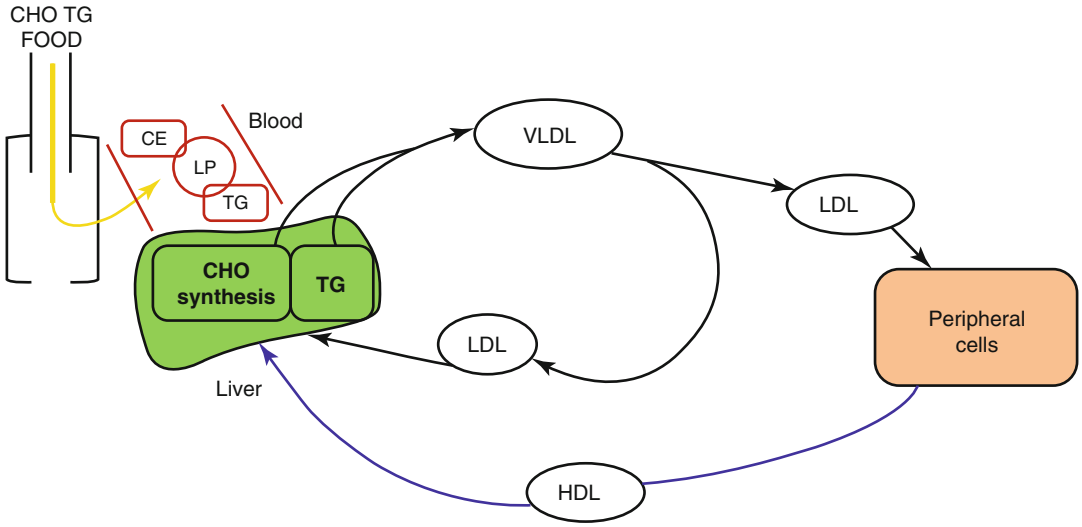

Fig. 12.2 Lipoprotein metabolism in unpregnant women

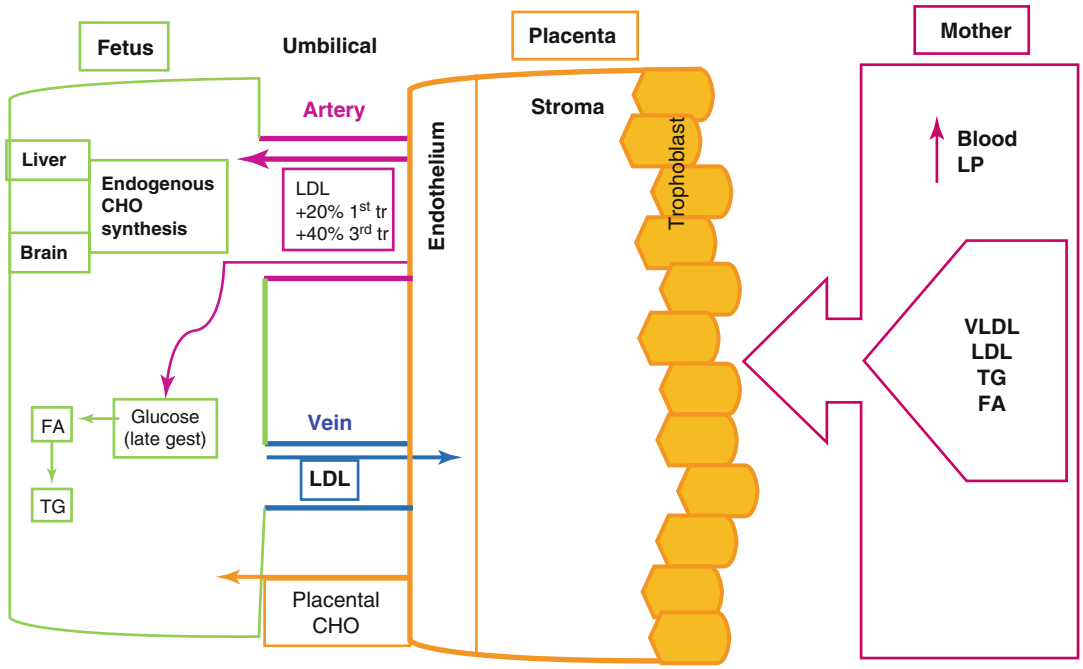

Fig. 12.3 Mother to fetus CHO and lipid exchange

accumulates fat stores thus providing nutrients sources to the fetus in the third part of pregnancy by the placenta transfer [22].

Fetal Cholesterol

Most of CHO required for the fetal growth is "*de novo*" synthesized by the fetus itself, thus making him autonomous from maternal or placental cholesterol supply [23]. The fetal CHO synthesis amount has been demonstrated to be higher than in adults and the endogenous production of CHO has been confirmed a mandatory need for fetal development [24]. The requirement of CHO is particularly high in brain and liver tissues, the synthesis by the liver being postulated to support other tissues requirement as demonstrated in animals [25]. Sterols markers of the CHO synthesis which include lanosterol, dihydrolanosterol, and lathosterol increase strongly since the 19th week of gestation while low till that period [26].

A maternal contribution to fetal CHO levels is anyway sustained by evidences supporting the postulate that up to 20 % of the sterol used by the fetus in the first trimester origins from maternal CHO and that an even greater percentage could be derived from the placenta with higher CHO concentrations [5]. More recently it was demonstrated that this figures grow up to around 22–40 % in the last trimester of pregnancy with a peak in the 2nd trimester [27] (Fig. 12.3).

Data, demonstrating that fetuses affected by CHO synthesis defect show at birth or later detectable CHO in tissues and plasma, support the hypothesis of a maternal supply to the fetal CHO pool [28, 29]. Furthermore comparing the LDL levels flux through the umbilical artery, which transports blood to the fetus via the placenta and that of the cord vein, which translates blood in the opposite direction (via the placenta to the fetus), it appears that LDL concentrations are higher in the umbilical artery [30]. CHO maternal LPs uptake can be also influenced by maternal, but not paternal, apolipoproteinE (APOE) phenotype thus adding a subject to exogenous CHO transfer to the fetus from maternal blood [31]. A further marker for maternal-fetal CHO transport consists of beta-sitosterol levels detectable in the amniotic fluid [26]. The maternal CHO availability in early pregnancy seems relevant to placental and embryonic development in humans [32–37] thus on the basis of the above demonstrations it has been hypothesized that the fetus can acquire maternal CHO whenever this hypothesis it is not definitively accepted and needs further confirmations.

CHO Transport

Maternal CHO has to cross the barriers between maternal and fetal tissues: the yolk sac in early pregnancy and, from approximately the fourth gestational week, the placenta (Fig. 12.4). Since the 10–12 weeks of gestational age the placental syncytiotrophoblast layer plug maternal blood on their apical side and fetal microvessels at its basolateral side. In-vitro studies suggest that the yolk sac is able to transfer externally derived CHO by receptor-independent processes such as aqueous diffusion [5, 38]. Most evidences come from studies conducted in animals that strongly suggest a transport over the yolk sac membrane during pregnancy [24].

Humans studies indicate that maternal CHO markedly contributes to the fetal CHO pool at early stages of gestation, both resulting significantly correlated [39]. The placental layer overcome by lipids could be more difficult. CHO uptake by syncytiotrophoblast cells is the result of receptor mediated as well as receptor-independent processes [24]. This step is allowed by means of different mechanisms, already demonstrated in animals, but still questionable in humans. These involve:

(a) LPs receptor mediated mechanisms involving LDL and VLDL receptors. LPs bound to the receptor undergo endocytosis to lysosomes/endosomes where CHO esters are degraded. The free CHO is transported across the cells via sterol carrier proteins, as Niemann-Pick C1-Like 1 (NPC1L1) and sterol carrier protein X and 2. The receptor is then recycled to the membrane [40].

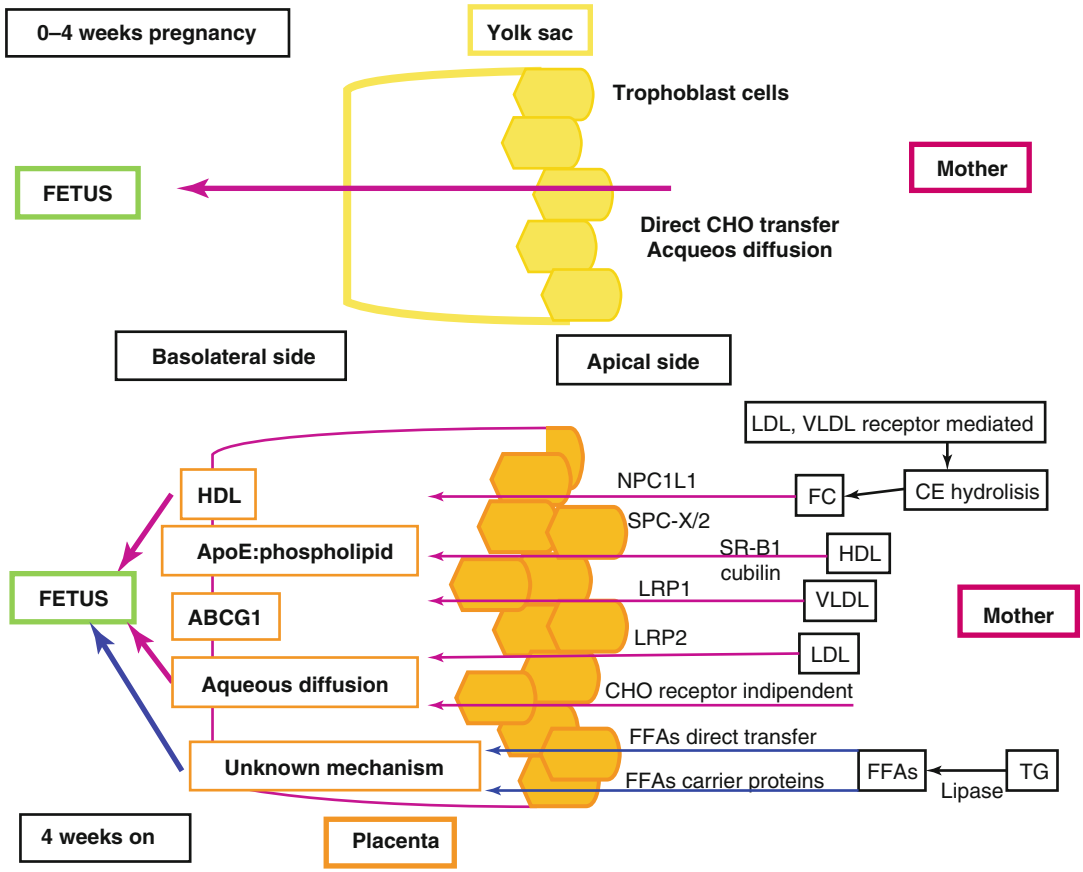

Fig. 12.4 Placental uptake and efflux of maternal LPs

(b) Receptors that transfer the CHO across the plasma membrane without internalization of the receptor. These include:
1. The scavenger receptor class B (SR-B1) that transports CHO mainly from HDL and with less affinity from LDL;
2. LDL receptor-related protein 1 (LRP-1) that binds apoE-containing particles such as VLDL. An increase in maternal blood CHO was demonstrated to reduce LDL receptor protein in trophoblasts and this result was considered a regulatory effect of maternal CHO on these receptor expression [41].
3. LDL receptor-related protein 2 (LRP-2), also named megalin, binds LDL and apoB, while cubilin binds HDL, apoE and apoA-1.

The efflux of CHO across cellular membranes is promoted by HDL through the ATP-binding cassette (ABC) transporters ABCG1, but contrasting data are available about ABCA1 [27, 42]. Moreover the efflux can be sustained by aqueous diffusion or apoE and phospholipid complex [24].

The placenta contains several LP receptors supporting its ability in taking up CHO from maternal blood but this subject is still uncertain as are the procedures that need to be explained [30].

Fetal Fatty Acids and Triglycerides

FAs can be synthesized "*de novo*" by the fetus, including some satured FAs and monounsatured

FAs originating from glucose. This appears particularly in advanced gestation when there is a gradual shift from embryonic to fetal lipids maternally derived [43]. The relevance of FAs in the fetal development is suggested by their maternal circulation increase during gestation [44, 45] and were shown to correlate with fetal lipid concentrations and fetal growth [46–48].

Fetal Fatty Acids and Triglycerides Transport

The energy provided by FAs is available from the maternal circulation in two different forms: FAs in their free form bound to albumin, or TGs transported as LPs (Fig. 12.4).

FAs are hydrolized by lipase activity that produces free fatty acids (FFAs). FFAs can also be directly uptaken from the maternal circulation then enter the placenta and trophoblasts cells through passive diffusion or by membrane carrier proteins to reach the fetus [13, 49–51]. When taken up by trophoblast cells FFAs are either transported transcellulary to the basolateral membrane, via an undefined mechanism, or utilized by the placenta itself for energy or as membrane substrates [6, 52].

Whenever LPs cross the placenta with difficulty [53], TGs could anyway be available to the fetus [6]. Maternal TGs carried by plasma LPs can be taken up intact by the placenta or undergo enzymatic lipase activity as shown in the placenta. Two lipases achieve FFAs from maternal circulating LPs containing TGs: the lipoprotein lipase (LPL) and the endothelial lipase. LPL shows the main relevant TGs lipase activity, is abundant in the human placenta and ensures that TGs are hydrolyzed into FFAs; the endothelial lipase is a phospholipase with little triacylglycerol lipase activity. FAs are then re-esterified to synthesize glycerolipids thus providing an energy reservoir in the placenta [52]. Glycerolipids undergo a further hydrolysis to allow FAs to be released into the fetal blood bounded to the alfa–fetoprotein to be rapidly transported to the fetal liver.

A relatively high placental transfer of lipids is related to the fetal requirement of some essential FAs to satisfy their increased request during gestation, as the fetus cannot synthesize them by itself. These are recognized as linoleic acid (LA), α-linolenic acid (ALA) and long chain polyunsatured fatty acids (LC-PUFA), in particular docosahexaeoic acid (DHA). LC-PUFA are mainly transported associated with plasma TG-rich lipoproteins as TGs rather than as FFAs, as demonstrated in humans [54]. DHA is particularly enriched in maternal plasma phospholipids (especially phosphatidylcholine) while ALA is present in TGs but not in phospholipids [55]. The correlation between maternal and late gestation fetal levels supports the mother to fetus transport of essential fatty acids with origins from the maternal diet and metabolism. An adequate availability of LC-PUFA to the fetus is clearly needed to preserve the normal fetal growth and this mechanism is preserved by the development of maternal hyperlipidaemia through pregnancy [6].

Fetal Lipoproteins and Molecular Mechanisms

Fetal LP levels in plasma were quantified during normal human gestation showing marked fluctuations (Table 12.1). The latter should be referred to the gestational age influence on fetal CHO levels with a strong inverse correlation. Early in the gestation Johnson [56] found that total plasma CHO levels were high thus suggesting a rapid biosynthesis rate of lipoprotein containing CHO occurring in the fetal liver (Table 12.2). As well CHO levels resulted significantly and directly correlated with maternal concentrations in fetuses younger than 6 months [6].

Table 12.1 Change in LP levels and gestational age

Week of gestation	CHO (mg/dl)	LDL CHO (mg/dl)	HDL CHO (mg/dl)
31–32	68.0 ± 7.0	44.0 ± 5.0	24.0 ± 2.0
33–34	73.0 ± 7.0	49.0 ± 6.0	24.0 ± 2.0
35–36	65.0 ± 7.0	35.0 ± 3.0	22.0 ± 4.0
37–38	64.0 ± 4.0	37.0 ± 3.0	23.0 ± 2.0
39–40	56.0 ± 2.0	30.0 ± 2.0	22.0 ± 1.0
41–42	53.0 ± 3.0	28.0 ± 2.0	22.0 ± 1.0

Human fetal adrenal glands make use of LPs containing CHO, and in particular LDL, as a substrate for steroid hormones production. Further LDL levels are inversely related to plasma concentrations of the major fetal adrenal secreted hormone: the dehydroepiandrosterone sulfate. The early CHO decrease in plasma fetal levels is concurrent with the adrenal gland size increase as happens through the 12–20 weeks of the gestation so providing a putative cause [57].

Some major variations characterize fetal and maternal LPs (Fig. 12.5). LDL and VLDL are poorly represented in fetal blood [58] while HDL represents the main lipoprotein class in cord blood [59, 60]. The fetal transport of CHO by HDL accounts at least the 50 % of the whole fetal pool, while LDL is the mainly transporter in the mother [61]. Fetal HDL differs from adult ones for physical-chemical properties. First the HDL_2 sub-fraction is mainly represented in fetal blood whereas HDL_3 sub-fraction is more prevalent in adults [62]. Second the fetal HDL apoprotein composition shows high apoE contents [62] and apoA-4 enrichment. ApoE is a relevant player in LP metabolism as interacts with cell surface receptors [63] and is mainly carried by HDL than TG rich LPs [61] so it is likely that the main role of apoE is to participate to the HDL metabolism. For instance apoE may facilitate the uptake of HDL by the fetal liver. ApoA-4 shows great structural similarities with apoA-1, which does not efflux CHO from the trophoblast [51], thus being postulated apoA-1 and apoA-4 to be exchangeable in the fetus [64]. The fetal HDL apoproteins profile gives an explanation for changes in their functions including an increased atheroprotective effect in the feto-placental vasculature. This effect could be explained by the role of apoA-4 and apoE in promoting lecithin:cholesterol acyl transferase (LCAT) activation [65]. To the antiatherogenic effect contributes the lower fetal colesteryl ester

Table 12.2 Fetal cholesterol levels through the gestational period

Week of gestation	CHO (mg/dl)
10–16 (n = 68)	85.4 ± 30.7
16.5–20 (n = 19)	39.9 ± 21.0
26.5–32 (n = 17)	67.8 ± 5.8
32.5–36 (n = 16)	58.8 ± 13.6
36.5–40 (n = 44)	51.4 ± 11.5

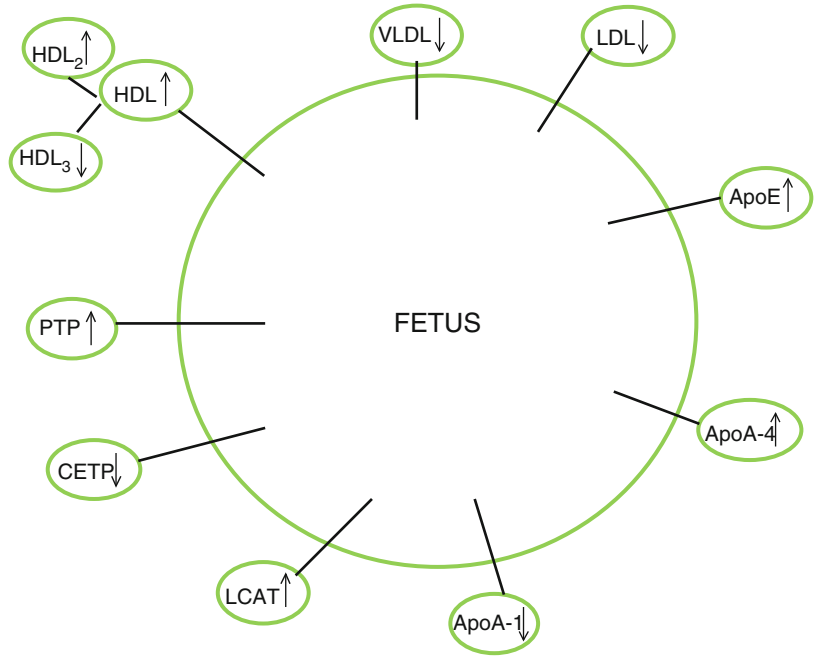

Fig. 12.5 LPs, apoprotein and enzyme activity variation in fetal plasma compared to maternal plasma

transfer protein (CETP) activity accounting for larger HDL subfraction [66] and the phospholipid transfer protein that increases particle's efflux capacity [67]. Fetuses exhibit major modifications of their HDL proteome in addition to the quantitative decrease of HDL-TGs and increase of HDL CHO levels [65].

FA transfer proteins expressed by the placenta include FA transport proteins (FATP1-4, FATP6), FA translocase protein (FAT/CD36), and plasma membrane fatty-acid-binding protein (FABPpm) [68, 69] which are devoted to secrete into the fetal blood the maternally derived FFAs [70]. FABPpm shows a selective action addressed to LC-PUFA transport to the fetus whenever in smaller quantity with respect to TGs.

Fetal genome and in particular APOE and LPL genes were demonstrated to modulate the maternal LP phenotype when particular polymorphisms change related are considered. In the presence of APOE2 isoforms the fetal liver uptake of HDL may be reduced [71]. Following metabolic changes include the depletion of intrahepatic CHO content and the reduction of LDL CHO concentration as occurs in postnatal life. Otherwise fetal genetic polymorphisms produce lipid phenotypes that are contrary to those observed in adults as the case of APOC3 (APOC3*S2 lowers LDL in fetus while increase LDL in adults). Furthermore the effects of fetal polymorphisms are strongly modulated by maternal polymorphisms suggesting that LP effects of these polymorphisms may differ before and after birth [33]. These data confirm a fetal contribution to maternal LP metabolism through the pregnancy [72]. Furthermore maternal genetic polymorphisms seems to impact on fetal LP levels, independently of maternal LPs and of fetal genome.

Fetal Consequences of Maternal and Fetal Disorders

The altered feto-maternal LP metabolism and exchanges exit in different degree disorders ranging from inability to the embrio implantation, leading to heavy malformations and abortion or minor disorders which impact on the future life. Disorders related are both of maternal or fetal origin (Table 12.3): the former include the Familial Hypercholesterolemia (FH), Pre-eclampsia and Diabetes; the latter the Intrauterine Growth Restriction (IUGR) and defects of CHO biosynthetic pathway.

Table 12.3 Fetal outcome related to fetal and/or maternal lipoprotein metabolism disorders

Maternal disorders	**Fetal consequences**
Familial hypercholesterolemia	High LDL CHO
	Preterm birth
Pre-eclampsia	Altered placental transfer of lipids
	High TGs
Gestational diabetes mellitus	LGA
	High FFAs
	Low TGs
Diabetes	Malformation and macrosomia
	High VLDL and LDL
Fetal disorder	
IUGR	Low CHO, LDL CHO, HDL CHO
	High TGs
SLOS	Central nervous system anomalies
	Deficient CHO

Maternal Disorders

Familial Hypercholesterolemia

Pregnant women affected by FH show higher CHO levels [73] than non-FH pregnant women whenever both show a similar percentage rise [15]. As well LDL CHO levels in the cord blood of FH newborns are higher if compared with non-FH newborn ones. On the contrary cord blood TGs and HDL CHO levels were similar to those of controls [74]. FH women gave birth to normal weight infants so indicating the good nutritional status of mothers and no impact on the fetal growth [75]. Furthermore epidemiological data indicate that maternal hyperlipidemia in pregnant FH women is associated with a more procoagulant profile and changes in fetal-uteroplacental

circulation, suggesting the fetus being at risk of preterm birth [76]. A study on over 2000 individuals showed that FH patients born from an FH mother present with higher LDL CHO levels than those who inherited FH from their father [77]. This observation open the discussion toward the atherogenic LPs profile of FH pregnant women, on deleterious effects on their offspring considering the risk of CVD later in life, and on the need of a treatment while on pregnancy of FH woman.

Pre-eclampsia

Pre-eclampsia (PE) is a multiple system disorder that affects the mother and can adversely influence the feto-placental unit. PE is associated with placental dysfunction, oxidative stress [78], endothelial cell activation [79], and it is a cause of maternal and fetal morbidity. PE affected women demonstrate marked dyslipidaemia, hypertension and an increased systemic inflammatory condition potentially triggered by widespread endothelial dysfunction.

PE pregnancy is characterized by a pro-atherogenic lipid profile with increased TGs levels, HDL reduction and increased small dense LDL particles [80]. This altered LP metabolism is involved in the pathogenesis playing a role in the development of the disorder. The placental vascular bed of PE pregnant women shows acute atherosis and atherosclerotic placental lesions characterized by the accumulation of foam cells and perivascular cell infiltration. These abnormalities reduce the placental perfusion and placental/fetal hypoxia may develop [78]. Fetal lipid metabolism can be affected due to an altered placental lipid transfer but contrasting data are described. Rodie et al. [81] observed that CHO levels were higher in the umbilical cord blood from pre-eclamptic pregnant women (with respect to controls) while HDL values were unchanged, supposing that placental transport mechanisms could be up-regulated. These results were not confirmed by Catarino et al. [50] who showed lower CHO and HDL values in the umbilical cord blood of PE pregnant women. This hypothesis is sustained by the evidence that LP receptor expression is decreased in the placenta of women with PE [82]. Moreover higher values of fetal TGs were observed in the umbilical cord blood associated with a significant increase in maternal blood TGs as a compensatory way to face the utero-placental hypoperfusion [83].

Diabetes

Gestational diabetes mellitus (GDM) and maternal diabetes mellitus type 1 or 2 are characterized by high incidence of fetal macrosomia and neonates that are large for gestational age (LGA) [84]. Poor metabolic control of diabetes early in pregnancy is associated with an increased risk of fetal malformations [85]. Poorly controlled diabetes in the second half of pregnancy could exit in neonatal macrosomia. Fetus from mother under poor metabolic control shows an LP cord blood level increase when compared with fetuses from non-diabetic mothers. This case frequently occurs in diabetic mothers.

Maternal hypertriglyceridaemia and hyperglycaemia are a consequence of the augmented insulin resistant condition, these changes enhancing the substrate availability to the fetus. In GDM pregnancies, maternal lipids correlate with fetal lipids and fetal growth; the increase of mother LP concentrations could have effects on lipids transferred to the fetus by their intensified passage [85]. This process enhances the risk for oxidative stress and lipid peroxidation [86]. Also in pregnancies under well-controlled GDM, both maternal TGs and FFAs levels have been shown to correlate positively with neonatal weight and fat mass, indicating that maternal hyperlipidaemia in GDM actively enhances the availability of lipids to the fetus, contributing to his fat depot accumulation. Maternal FFAs and TGs levels predict also LGA birth weight and these values are linked with those measured in cord blood serum [48].

Fetal Disorders

Intrauterine Growth Restriction (IUGR)

Small for gestational age (SGA) newborns could be divided into two groups, depending on the causes of low birth weight. SGA neonates include those who are genetically small and IUGR. In this

latter pathologic condition the fetus does not reach its genetically growth potential, with a growth velocity reduction and related fetal disorders. The current hypothesis about pathogenesis include the insufficient trophoblast development that may lead to atherosclerotic placental lesions. Thus IUGR has similar placental pathology as PE [87].

Authors evidenced lower maternal LDL and CHO concentrations in pregnancies complicated by IUGR but few data are available about fetal LPs in IUGR condition. Pecks et al. [88] showed a significant decrease of HDL and LDL CHO levels in the umbilical cord blood of IUGR pregnant women. Furthermore authors underlined the current increase of oxLDL/LDL ratio which was negatively correlated to HDL concentrations. On the contrary TG levels were significantly increased.

IUGR fetuses show a proatherogenic profile. Based on Baker's hypothesis, change in lipid concentrations may represent one of the pathogenic links between low birth weight for gestational age and subsequent cardiovascular events in adulthood. This suggests a metabolic programming in intrauterine environment resulting from placental insufficiency [88].

Defects in the Cholesterol Biosynthetic Pathway

Seven known disorders involving enzyme defects in post-squalene cholesterol biosynthesis have been identified: desmosterolosis, X-linked dominant chondrodysplasia punctata, CHILD syndrome, lathosterolosis, hydrops-ectopic calcification-moth-eaten skeletal dysplasia, Antley–Bixler syndrome, Smith–Lemli–Opitz syndrome. The most common is the Smith-Leimli-Opitz syndrome (SLOS), while the other six syndrome are extremely rare and often lethal.

SLOS is a congenital multiple anomaly/intellectual disability syndrome caused by a deficiency of CHO synthesis resulting from an inherited deficiency of 7-dehydrocholesterol (7DHC)-reductase, encoded by DHCR7 gene. The enzyme catalyses the last step of CHO biosynthesis, the conversion of 7DHC to CHO. As a result deficient CHO levels are produced while the precursor 7DHC and derivatives accumulate both during embryonic development and after birth. Tissues (especially brain) deprived of CHO, or because of sterol precursors and derivatives deposit, develop abnormally and function poorly. Substitution of 7DHC for CHO alters the lipid raft stability, protein compositions and decreases membrane bending rigidity. The precocious altered sterol composition in SLOS affects the physical and chemical properties as well as the function of cellular membranes. These changes are causative of signal transduction [89]. IUGR is the most frequent ultrasound finding, detected in 67–100 % of affected fetuses [90, 91]. SLOS affected newborns have a distinctive appearance with specific facial dysmorphism and suffer from multiple congenital anomalies including cleft palate, congenital heart disease, genitourinary abnormalities, and malformed limbs. They often manifest mentally retardation with significant central nervous system anomalies [92, 93].

Conclusion

Lipoprotein metabolism in human fetus is uncompletely understood but it is finally clear that a strict relationship between mother and fetus LP phenotypes effect pregnancy outcome and mother and fetus well-being. This correlation is related to the genetic fetal and maternal background and LP polymorphisms associated, besides environmental conditions.

Main key points concern the relevance of fetus CHO pool and FAs that are critical to the growth rate, the fetus as auxotrophic human being for CHO and lipid synthesis and the LP transport as feasible from maternal blood through the placenta. CHO transport in the fetus is mainly supported by HDL subclass and among FAs LC-PUFA uptake by the placenta is preferential. The transport is made available by transporters or by acqueous diffusion including both CHO and FAs. Considering the CHO efflux from the placenta to the fetus this is mainly provided by HDL or by simple diffusion while mechanisms regarding FFAs efflux are not finally established. Further studies aimed to ascertain actually unrecognized physio-pathological mechanisms are requested whenever ethical issues should not be neglected.

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Part III

Gene and Human Fetal Development up to Second Trimester

13 Gene Regulatory Networks and Epigenetic Modifications in Cell Fate Decisions During the Early Embryonic Development

Siddhartha Roy

Introduction

The fertilized zygote is totipotent, capable of differentiating into all the cell types present in an adult body as well as extra-embryonic tissues required for embryo development. The fertilized totipotent single cell divides, giving rise to the multi-cell morula and later the blastocyst. By the morula stage, the first differentiation events occur, giving rise to the trophectoderm and the Inner Cell Mass (ICM) [1]. Trophectoderm later gives rise to the trophoblasts, while the ICM develops into the embryo. The inner cell mass has a more restricted potency than the zygote in that it cannot give rise to trophoblast cells. Thus, these cells are referred to as pluripotent [2]. By the late blastocyst stage the inner cells mass further differentiates into the epiblast and the primitive endoderm layers (Fig. 13.1) [1]. Further down the embryonic development road, the epiblast gives rise to the germ layers, three layers of further differentiated cells, the ectoderm, the mesoderm and the endoderm that ultimately give rise to all the cell types in the adult animal. Differentiated cells that originate from the zygote do not normally cross the lineage boundaries. Thus, they become committed to their lineage and restricted in potency.

How this continued restriction of potency occurs is not fully clear at the molecular level. However, recent path-breaking research is pointing toward primacy of gene regulation in this process [3, 4]. It has been known for a long time that the DNA sequence of the genome is identical in the totipotent cell and all the differentiated nucleated cells. Thus, different stages of cell differentiation differ only with respect to expression of genes. First conceptual model of cell differentiation was proposed by Waddington [5]. In this model, the pluripotent cells sit on top of a mountainous landscape, which describes some sort of downward gradient of potency. As the cell differentiates, the cells on the top of the mountain roll down and settle into various local minima; rolling down being equivalent to loss of potency and differentiation (Fig. 13.2). Although this model had an iconic influence in this field, it is largely metaphoric without giving any hint to the actual underlying mechanisms. In this article, we will explore how recent elucidation of regulation of gene expression and epigenetic regulation is throwing light on molecular mechanisms of cell differentiation at the early stages of development.

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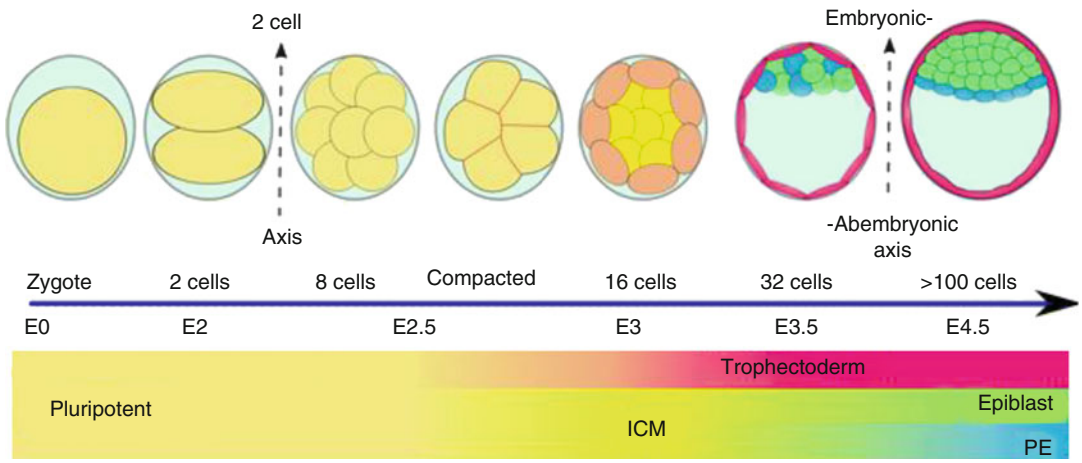

Fig. 13.1 Morphological and lineage specification steps that occur during early mouse embryonic development (The figure is reproduced from Krupinski et al. under Creative Common License [54])

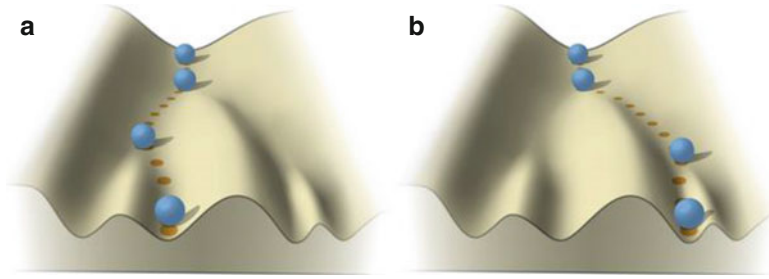

Fig. 13.2 (**a**, **b**) The Waddington's landscape of cell differentiation (The Figure has been reproduced from Mitchell et al. under the Creative Common License [55])

Gene Regulatory Networks

Pioneering work by Gurdon and others on nuclear transfer indicated that differentiated cell nucleus contains all the information to revert back to a pluripotent state [6]. Much later, Yamanaka and co-workers created induced pluripotent cells from differentiated cells by expressing a small number of relevant transcription factors [7]. Thus, it is now well established that it is not the sequence of genes per se, but its expression that is the key to inducing a particular differentiated cellular state. A particular pattern of gene expression results in a particular cell type and this pattern of expression may be heritable over cell generations. Thus, in order to understand how cells differentiate, we delve into the present level of understanding of eukaryotic gene regulation.

Broadly, gene expression is primarily regulated at two different levels. At one level, genes are regulated by proteins that bind in a DNA sequence specific manner, called transcription factors [4]. The activity of a transcription factor dictates the state of expression of a gene that it regulates and in combination with other transcription factors determine the overall pattern of gene expression. This type of regulation is not heritable across cell generations. The second type of regulation occurs through modifications of DNA itself and its associated proteins and is one of the major mechanisms of the epigenetic regulation. The latter is often heritable across cell generations, giving a sense of quasi-permanency. The two different modes of gene regulation are not mutually exclusive and often deeply interrelated. Binding of transcription factors often lead to recruitment of chromatin modification machineries and consequently to changes in status of epigenetic modifications. Similarly, changes in chromatin modification may lead to

changes in accessibility of transcription factor binding sites. Thus, the two modes may be considered complementary and as two sides of the same coin.

Transcription factors rarely work alone in eukaryotes. Unlike in prokaryotes, eukaryotic gene regulation occurs through combinatorial binding of several transcription factors in the upstream regulatory regions of genes [8]. It is becoming apparent that one of the fundamental reasons for this phenomenon may lie in the fact that eukaryotic transcription factors by themselves lack sufficient DNA sequence specificity for the target site. The transcription factors have to operate and specifically recognize a small piece of DNA sequence in the backdrop of more than a billion non-target basepairs in the genome. This would require DNA sequence discriminatory ability of a very high order which is not reached and perhaps is not attainable by an isolated transcription factor [9]. Eukaryotes reach this level of discrimination by combinatorial binding of various transcription factors in the same gene regulatory region. Thus, we usually observe multiple transcription factors cooperating with each other, forming a network of gene regulatory proteins. Together with the genes they regulate and other involved players, they form the gene regulatory network [10]. Gene regulatory networks have the general characteristics of networks in general and may have different topologies. It is difficult to discuss the types and properties of gene regulatory networks in detail here and the reader is referred to some excellent literature on this [11–13]. However, we will focus on one relevant property which we think is crucial for the understanding of cell differentiation.

Gene regulatory networks are critical for understanding molecular nature of cell differentiation and development. External signals in the form of cytokines, positional information etc., are transduced through signaling pathways to gene regulatory networks. The gene regulatory networks are the final executors of cell fate decisions and within them one or a limited number of transcription factors, the master regulators, play a dominant role [14, 15]. These cell fate determining gene regulatory networks are required to exist in steady-states (changing concentrations will also change the occupancy of promoters and hence the gene expression) so as to dictate a particular pattern of gene expression. Existence of steady-states is dependent upon the nature of the network architecture [16, 17]. Many biological networks have architecture that fall into the class that contains a positive circuit [18, 19]. Having positive circuits is the necessary condition to have multiple steady-states, that is, possessing the property of multi-stationarity [20]. For gene regulatory networks that determine the cell fate decisions, access to multiple steady-states leads to the possibility of expressing multiple patterns of gene expression. Steady-states are important as in these states the concentrations of the transcription factors remain more or less invariant, leading to a stable pattern of gene expression. Expression of different combination of genes is possible if the target sequences have different affinities and change of activity of the transcription factor(s) will lead to different fractional occupancies and consequently expression of different genes. Such different classes of target sequences and their biological consequence have been described for transcription factors such as p53 [21]. Thus, external signaling leads to changes of transcription factor concentrations in the nucleus which leads to a different steady-state of one or more gene regulatory networks, resulting in different transcription factor occupancy and changed pattern of gene expression (Fig. 13.3). Clearly, understanding the gene regulatory networks that execute the cell fate decisions are of prime importance for understanding cell differentiation.

Chromatin Modifications and Cell Differentiation

As pointed out before, change in gene expression pattern as a consequence of changes in gene regulatory networks (i.e. resulting from changes in transcription factor occupancy) does not lead to heritable gene expression patterns per se. Resistance to change of gene expression patterns in response to sudden alterations in signaling

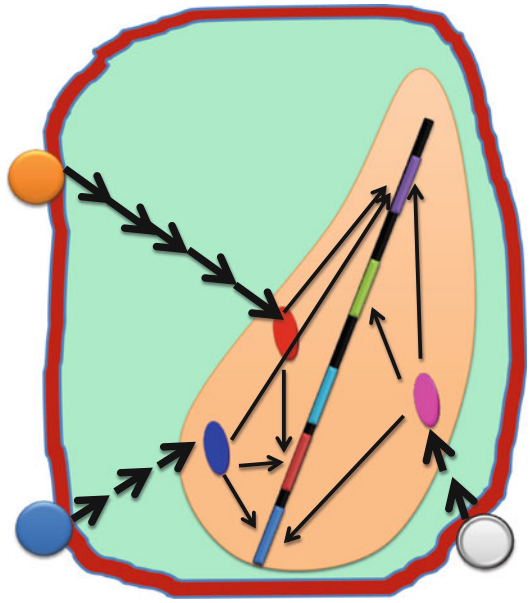

Fig. 13.3 A schematic figure of how external signals are transmitted to transcription factors via signal transduction pathways. The signal causes activities of relevant transcription factors to increase in the nucleus causing change in promoter occupancy of transcription factors of various genes. The *oval* shaped objects are the transcription factors, the *arrows* represent signal transduction pathways and the *round* objects represent the signaling ligands occupying respective receptors

pattern or stochastic fluctuations and its heritability across cell generations are important factors for maintaining the quasi-irreversible nature of lineage committed or differentiated cells. Such stability and heritability is offered by epigenetic chromatin modifications, which are thus essential components of lineage commitment and cell differentiation [22, 23].

Chromatins undergo a large number of modifications including that of the DNA and associated proteins [24]. Among the modifications, DNA methylations and histone acetylation and methylations are most well studied. DNA methylations are used to silence genes quasi-permanently [25]. Histone modifications are generally used as softer marks, allowing reversions under certain conditions. Apart from their direct effects on transcription, the chromatin modifications also are important players in changing the way the chromatins are condensed, leading to further regulations of gene expression. Two major regions of chromatins which show variations in condensation properties can be readily identified: The condensed heterochromatin, containing mostly transcriptionally silent genes and the less condensed euchromatin and containing regions rich in genes that undergo active transcription. Some heterochromatin regions are fixed in all cell types, whereas some others vary from cell type to cell type. Thus, accessibility of genes to transcription factors and machineries could be restricted in the condensed heterochromatin segments, adding another layer of regulation [26].

Upon fertilization and formation of the zygote the genome undergoes de-methylation and by the morula stage it has reached the minimum. Re-methylation then starts. Beyond the initial developmental stage, further differentiation is accompanied by rewriting of epigenetic marks, suggesting that this form of regulation of gene expression plays a vital role in cell differentiation in the early developmental stages [27]. The precise mechanism of how epigenetic marks affect cell differentiation is still not well understood. Similarly, the relationship between the cell fate determining gene regulatory networks and the epigenetic regulation is not quite clear. In a previous article, we have attempted to connect these two modes of gene regulation and suggested that epigenetic marks restrict accessibility to certain steady-states of cell fate determining networks, thereby channeling cells to a particular lineage committed state [28]. Thus, a fuller understanding of cell fate determining gene regulatory networks, epigenetic gene regulation and their interrelationship is vital for elucidation of molecular mechanisms underlying early development.

Embryonic stem cells are derived from the inner cell mass of an embryo. Embryonic stem cells have been instrumental in developing many of our ideas about the pluripotent state and its lineage commitment. Their chromatin is believed to be in a hyper-dynamic state with very few regions in hetero-chromatinized state [29, 30]. Probably, as a consequence they are able to switch between different gene expression programs and they appear to do so in vitro, stochastically [31, 32].

Transcription Factors Involved in Differentiation of the Zygote

As mentioned above, the first segregation and differentiation event occurs in the morula stage. The trophectoderm and inner cell mass lineages segregate, with former giving rise to trophoblasts and the latter forming the epiblast and the primitive endoderm layers. The inner cell mass is pluripotent and it appears that the state of pluripotency is initiated and maintained largely by three transcription factors, Nanog, Oct4 and Sox2 [33]. It appears that Nanog also plays a central role in the differentiation of ICM [34]. Nanog belongs to the homeodomain class of transcription factors and has been used in induction of pluripotency from the differentiated cells. Oct4 and Sox2 are also important components of pluripotency maintenance [35]. Oct4 belongs to the POU family of transcription factors, whereas Sox2 has the HMG box binding domain. Oct4 and Sox2 bind to many promoters of pluripotency genes together with cooperativity.

The first differentiation step from zygote to trophectoderm and inner cell mass is dictated by a limited number of transcription factors [36], of which Oct4 and Cdx2 (dominant determinants for ICM and TE lineages, respectively) were thought to be the master regulators of cell fate; Oct4 priming the ICM lineage and Cdx2 priming the trophectoderm lineage [37–39]. They were reported to inhibit each other's function reciprocally, thus maintaining a balance [38, 40–42]. Appropriate signals may increase activity of one or the other, tilting the balance in favor of a particular cell fate. These could be the crucial components of the master regulatory switch regulating the earliest cell fate decision. However, some recent studies have raised doubts about the primacy of these transcription factors in the cell fate determination and other transcription factors may be involved. A very recent study has suggested that Arid3a could play important roles for determining the cell fate to trophectoderm lineages. Thus, it appears that a network of a number of transcription factors are involved in priming the cell fate decisions at this stage. A full elucidation of the network and its properties may be a very important goal in the near future.

Epigenetic modifications also play crucial role in lineage commitment of trophectoderm and inner cell mass [43]. Most notable among them is the histone methyl transferase, SETDB1 [44, 45]. In mouse, SETDB1 expression occurs in the inner cell mass which is initiated at the blastocyst stage. It methylates H3K9 and thus is a repressor of gene expression. It interacts with Oct4 and recruited by it to the appropriate sites. The targeted genes are likely to be involved in lineage commitment process of trophectoderm, such as Cdx2. The multiprotein complex NuRD [46] is also an important component of maintaining inner cell mass identity and prevents lineage commitment to trophectoderm. Embryonic stem cells containing defective NuRD shows inappropriate expression of trophectoderm specific genes such as Elf5 [47]. NuRD deficient embryonic stem cells can be easily converted to trophoblasts cells, suggesting that it is part of a barrier that prevent inappropriate conversion to trophectoderm lineage. Many other histone modifiers and chromatin remodelers are involved in the survival of inner cell mass, including Tip60 and MOF [48]. Clearly, the histone modifiers and chromatin remodeling proteins play an essential role in the early development.

Differentiation of Inner Cell Mass into Epiblast and Primitive Endoderm

The inner cell mass at the blastocyst stage differentiates into the epiblast and the primitive endoderm layers. Just prior to implantation, the inner cell mass starts segregating into two populations: The progenitor of the epiblast cells and the progenitors of the primitive endoderm cells. Recent studies suggest that two transcription factors play lineage determining roles, Nanog and Gata6. These two transcription factors seem to express in a mutually exclusive manner in the inner cell mass cells, with the cell expressing Nanog becoming the epiblast and cells expressing Gata6 proceeding to primitive endoderm. Although, these two transcription factors appear to be the master regulators in the cell fate determination at the inner cell mass level, there are other transcription factors involved in the gene regulatory network [49, 50]. It appears that Gata6 progressively activates Sox17, Gata4 and Sox7 [51,

52]. The segregation and differentiation of primitive endoderm and epiblast is coupled through the Fgf4 and Fgf receptor pathways [53].

Discussion and Conclusion

A major component of early development is lineage commitment of totipotent zygote to more differentiated cell types. We have described some of the underlying principles of this lineage commitment. Although, the principles are not fully elucidated, it is clear that the gene regulatory networks play the master regulatory role. Although they initiate the process of lineage commitment upon receiving the appropriate signals, the final execution depends on many other players acting downstream. Most important among these players are the enzymes that modify the chromatin components, particularly histones and DNA, which results in change of conformation of the chromatin. These changes result in a more permanent state, defining a differentiated cell type. All the players in either the regulatory networks or the chromatin modifiers are not fully known. However, we have described some players that are involved in fate decisions of the more differentiated cell types that emerge in the early development and expect that in the near future a full catalog of players involved will be known. It should then be possible to fully construct the gene regulatory networks, which should allow us to lay a fuller molecular and systems level understanding of the early development process.

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14 Personal Human Life Begins with the Formation of Adult Type Hippocampus at 13th Week of Development

M. Kemal Irmak

When Does Personal Human Life Begin?

One of the most controversial topics in modern bioethics, science, and philosophy is the beginning of personal human life (personhood). Many philosophers and scientists have argued about the definition of personhood and the time for the beginning of a personal human life, however an acceptable explanation has not been provided yet. The consequences of this discussion are vitally important, as they may help to articulate more adequate arguments on some bioethical issues, like the definition of the moral status of embryos, abortion and embryo research. The purpose of the present chapter is therefore to propose when, in the course of normal development, a personal human life begins.

The availability of embryonic stem cells may open novel avenues for medical treatment of otherwise incurable diseases [1]. Embryonic stem cells are able to make any cell except placental cells and are also immortal [2]. However, the generation of human embryonic stem cells sometimes requires the destruction of early human embryos. This raises the same ethical questions and conflicts that are often heard when the ethics of abortion are discussed. Some people regard an embryo to be a full human person; it has all of the rights of any citizen – including the right to life; thus any procedure that injures or kills an embryo is murder of a human. Most people however regard the beginning of individual human life to occur much later in pregnancy; thus, killing a recently fertilized embryo is not murder of a human person [3]. What is the status of the embryo at several days old? Is the embryo alive? Yes, from its beginning the embryo is cellular and it is alive, no one questions this. But, is the embryo human? If we could catch the embryo before it reaches a stage of being judged human, we would take the embryonic stem cells without any concern. Seeing that monozygotic twinning can occur as late as day 14 after conception and such identical twinning will produce two individuals with different lives, this could be proposed as a pre-embryonic stage where the single individual person is not yet fixed [4]. Today, embryo research is allowed up to 14 days in United Kingdom after which the splitting and the forming of twins is no longer possible [4].

Most religious traditions hold that what makes one a person is the possession of a soul [4, 5]. When the body meets with the soul, it comes to be a human person, with all the attendant rights, especially his basic right to life. However, the exact gestational age at which ensoulment is believed to happen is debatable. Common views include ensoulment happening

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at the moment of conception, at the formation of brain and at the time of viability [5, 6]. In all religious groups abortion has always been regarded as sinful. However, for many centuries the termination of a pregnancy at an early stage carried lesser penalties than one later. This was related to the view that the human soul does not enter the embryo until 40 days or so after conception [5, 7]. Most religious groups thus, make a distinction between the moral status of the unformed and formed embryo, and think of the human person in the full sense, coming only with a delayed ensoulment [6].

Brain Cortex and Personhood

What makes human beings human and when does personal human life begin? From a medical point of view the function of the brain is fundamentally linked to being human. The brain controls almost all functions of the body and determines its psychological makeup [6]. Legal definition of death is based on the absence of brain activity, even though all the rest of the body is viable. Children born with anencephaly can never live a personal human life. Indeed, a living body without a brain would be a mass of cells without personhood. A conglomeration of cells in the early phase of pregnancy can hardly be characterized as a human person. Therefore personhood cannot occur until it has something that counts as a brain. The human identity, personality, and worth is associated with the functioning of the brain, so only when the brain is developed can there be any talk about an unborn human being [8].

In elaborating on this theory, Goldenring [9] defended the 8-week mark as the starting point for personal human life, indicating in his view the point at which there is integration of the brain as a whole. Kushner [10] argues that the initiation of brain activity is the most reasonable time at which to fix the start of life. Similar arguments are put forward by Shea [11] who recognizes that a new human life comes into being when the newly developing body organs and systems begin to function as a whole under the direction of a functioning brain at 8 weeks of gestation. Emphasis is placed on a point during development when brain tissue begins to function. Brain death is the cessation of function of a brain; brain birth is the very gradual acquisition of function of a developing neural system. Is this developing neural system a brain? At some point, it must become a brain, but at what point? In particular, can we legitimately call it a brain at around 8 weeks' gestation?

The fetal brain develops very gradually over time from a comparatively simple to a more complex structure that comprises a number of functionally differentiated neurological components, including the critical cortex. Brain cortex is the outer layer of cerebral hemispheres and it is responsible for sensory perception, movement, language, thinking, memory and consciousness [12]. Brain cortex is also necessary for the personhood [6, 13]. The development of different parts of brain begins at different stages in fetal development [14, 15]. The structure of the adult cortex is highly complex and specialized and a great deal of change must occur during fetal development before an adult-like cortical structure is recognizable. However, the cerebral cortex is an extremely rudimentary structure at 8 weeks of gestation on which so much emphasis is placed [15]. At this point, we must remember the existence of two different types of cerebral cortex and two different stages of cortex development. The bulk of the cerebral cortex is a six-layered structure called neocortex (young cortex). The remaining of the cortex around the hilus of the cerebral hemisphere is known as allocortex (other cortex) [12]. Allocortex has a relatively elementary structure with three basic layers and is composed of the olfactory cortex and hippocampal formation [12].

The neocortex undergoes a long period of differentiation and maturation and neocortical 'life' does not begin while the neocortex is getting organized [15]. Neocortical birth could be located after the 24th weeks of gestation when cortical organization in the fetus begins to resemble that of adult type [15]. During the early fetal period and midgestation, the individual neocortical layers are not easily identifiable since there is a

continuous influx of new neurons into the cortical plate and this plate does not contain synapses until 22–24 weeks of gestation. The six-layered adult laminar pattern gradually appears after 24 weeks of gestation owing to the progressive differentiation of the cortical plate [16–18]. Thus, neocortical birth could be located at the occurrence of the first minimal level of structural organization of neocortex which starts after the 24th week of gestation [19].

Hippocampal Development

The term allocortex is applied to the part of the brain that consists of a rim of cortical tissue around the hilus of the cerebral hemisphere [12]. It is the first part of the cortex to initiate differentiation from the eighth week onward and it forms a continuous, almost circular strip on the medial and inferior aspects of the hemisphere before 13th week of development [20–22]. There are two allocortical formations in the mammalian brain: the hippocampal formation and the olfactory cortex [12]. At first, olfactory cortex occupies a large part of the basal aspect of the brain. Later in development, the olfactory cortex becomes restricted to a small part of the brain and hippocampus becomes the main structure of the adult allocortex [23, 24].

Hippocampal formation is a large C-shaped allocortical structure in the medial aspects of the temporal lobe [12]. It begins to develop by 8-week gestation and one of the earliest sulci identified by fetal MRI is the hippocampal sulcus [25]. In the ninth week, the hippocampal primordial can be identified on the medial aspect of the developing cerebral hemisphere [26]. The hippocampal formation of adult type may be recognized at MRI of 13th week of development [26]. Most pyramidal cells are generated by the 13th week [27] and the subsequent period is characterized by an increase in the volume of the hippocampal formation. As most of the pyramidal cells have already formed and are currently undergoing the process of differentiation, the cellular components that increase in number thereafter are mainly of glial origin [26]. Hippocampal connections also develop prior to neocortical pathways, and reciprocal entorhinal–hippocampal projections are among the first cortico-cortical connections to be established in the human brain [28].

Hippocampal Birth at 13th Week of Development

The most striking feature of the early human fetal brain is the differentiation of hippocampal formation, and the intense expression of functional proteins in these areas [29–32]. As seen in the previous section, hippocampal cortex reaches its final structure at an earlier stage than the neocortex, and it is already developed [20–22, 25, 26, 32–34] at 13th week of development. From now on, the macroscopic aspect of the hippocampus remains unchanged until birth, and its morphology in the early fetal period, neonatal and pediatric periods is quite similar [20, 26]. Thus, three-layered adult laminar pattern of hippocampus appears around 13th weeks [21, 22] and hippocampal birth could be located at the occurrence of the first minimal level of structural organization of hippocampus at the 13th week of development.

Hippocampal formation plays a key role in memory and emotion. It acts as a comparator of novel and familiar stimuli and in the initiation or inhibition of behavioral strategies as appropriate to the situation. It therefore becomes linked to emotions such as anxiety and to setting appropriate motor responses [29]. Elaboration of hippocampal functions is also associated with the development of self-awareness, consciousness of the self [9], and hippocampus seems to be essential for the preservation of identity [12]. Thus, hippocampus can be regarded as the seat of emotion, memory and personhood.

We propose here a hippocampal birth theory suggesting that a personal human life or personhood cannot begin until the development of the hippocampus. Hippocampus is reliably present at about 13th weeks' gestation, and formation of hippocampus would indicate that the second level of life has commenced, and therefore could be taken to herald the beginning of a personal

human life. We can say that the informational capacity of the zygote and early embryo is not sufficient to direct the development of anything personal, and is not sufficient to constitute a genetically stable subject as a human being. At the 13th week, fetus has distinctive human characteristics and possesses the primordia of all the internal and external organs and parts. An adult type hippocampus also emerges at about 13 weeks' gestation, and the fetus could be considered a personal human being starting then. Hippocampal birth, the emergence of a mature hippocampus is therefore proposed as a reasonable time to demarcate the beginning of personal human life (personhood). Prior to 13 weeks, existence is limited to cells, organs, and organ systems which have the potential for integration into a full human organism. Standardization and widespread acceptance of a definition of "hippocampal birth" would potentially clarify many medical and legal questions regarding elective abortion and embryo research.

There must be a point during development following fertilization at which it is reasonable to claim that the organism changes from nonperson to person. When does this special point in development occur? Seifert treats the human body as a composite of a biological organism and an intellective soul [35]. If we accept that the soul essentially has the capacity for personhood, it seems that the soul does not begin to exist until there occurs an appropriate seat for the soul in the fetal brain. This position would seem to require that the immortal soul only be infused into a fetus with sufficient cortical development. We suggest therefore that the soul is added to the already-existing physical body, when the hippocampus is formed at 13th week [36] even in a pregnancy occurred by self-fertilization [37, 38]. Prior to this point, there exists a cluster of biological cells which has the potential to develop as one human organism [39]. This alternative is compatible not only with the facts of modern medicine but also with the traditional understanding of ensoulment defended by many theologians who argue that the peculiarly human soul is not incarnated until there is appropriately an organized matter [1].

Soul Must Have a Material Component

Mental functions are powers that the soul has exercised by means of the physical entity called the brain [40]. A question is raised: "How is it possible for an immaterial substance to interact with a physical body?" [40]. No experimental data can be sufficient to bring us to the recognition of a soul, but there must be a substance as the basis of personal identity, for without space-occupying substance, there would be no way to account for the soul's ability to interact with the body [41, 42]. It was suggested that the soul substance consists of cosmological dark matter [43]. The dark matter is a universal connecting medium, filling all space to the furthest limits, penetrating the interstices of the atoms without a break in its continuity. So completely does it fill space that it is sometimes identified with space itself, and universe is built up in this fluid and move through a sea of it [43]. Astrophysical observations indicate that dark matter constitutes most of the mass in our universe, but its nature remains unknown [44]. It is called dark matter since it neither emits nor absorbs light. The existence of dark matter is inferred by its gravitational effects on ordinary matter and radiation [45, 46]. With the conception that the dark matter is the primary form of all substance, that all other forms of matter are merely differentiations of it, then it seems that soul substance which is in this life linked organically with the body can be identical with the dark matter. The soul is likely to work into man's physical body directly via that dark matter [43]. Evidence of the existence of dark matter has been found in large high-energy particle accelerators at CERN and Fermilab (Illinois) [47].

Vomeronasal System as a Point of Entry for the Soul Together with Dark Matter

We thought that while the soul has a material component (dark matter), there must be an open window to the brain for the entrance of the soul

with dark matter. In this respect, vomeronasal organ (VNO) which is found in the nasal cavity and which has connections with the brain only between the 12th and 14th weeks of human development – a period including the time of ensoulment at the 13th week – seems to be the most appropriate window through which the soul and dark matter can enter the brain [48]. Actually, VNO is said to be the place in the body where the nervous system is closest to the external world [49]. Sensory cells of the vomeronasal system (VNS) are located within the sensory epithelium of the VNO, bipolar in shape with a single dendrite and an axon originating from its soma. The dendrite reaches the surface of the lumen of the VNO to form a dendritic terminal that bears microvilli [50]. The axon leaves the sensory epithelium, forming the vomeronasal nerve with neighboring axons, traveling toward the brain, and terminate on dendrites of second-order neurons in the accessory olfactory bulb [49, 50]. Axons of the second-order neurons of the VNS make close connections with the amygdala and hippocampus [49–51], the seat of emotion, memory and personality; shortly the seat of the soul.

The vomeronasal organ is a fluid-filled, tubular structure located at the base of the nasal septum that opens into the nasal cavity via a duct at its anterior end [52]. It is a chemoreceptive structure with direct axonal connections to the accessory olfactory bulb in many terrestrial vertebrates [53]. Pheromones presumably bind to the vomeronasal organ and exert behavioral or physiologic responses, thereby allowing chemical communication between animals of the same species [54]. The effects of pheromones are thought to be mediated by signals from the accessory olfactory bulbs to the amygdala and hypothalamus [52]. The vomeronasal system is well developed and functional in adult animals [55], while human VNO becomes rudimental before birth [56]. VNO in the human embryo contains bipolar cells similar to the developing vomeronasal sensory neurons of other species, but the structure becomes more simplified later in development [57–63], having no obvious way of communication with the brain. In humans the VNO, including the vomeronasal nerve and associated ganglion cells, is first recognizable at eighth week of development [64]. VNO is well developed during the 12–14th weeks of development [48], but VNO loses receptor cells and becomes a ciliated, pseudostratified epithelium after 14th week of age [65, 66]. Moreover, the vomeronasal nerve connecting the vomeronasal organ with the accessory olfactory bulb (AOB) degenerates between week 14 and 28 [67, 68] leaving the function of the human VNO unclear. The AOB which is a primary brain center for the VNS [51, 62], is present in human embryos and certain stages of fetuses, but becomes degenerated and it is not identifiable after 7 months [69]. These observations support the view that vomeronasal system functions mainly during the intrauterine period in humans, especially during the period of ensoulment [36, 70]. Thus the development of the vomeronasal structures seems to be limited to a restricted time frame in humans, when they play a role for the ensoulment [59]. It was proposed therefore that the human VNO has functions mainly during fetal development when the VNO, along with the vomeronasal nerve, contributes to the transfer of the soul and its dark matter to their proper sites in the brain [71].

Conclusion

Above considerations make it appear likely that the hippocampus is the primary center harboring the soul and the human life (personhood) begins at the 13th week of development with a delayed ensoulment. The soul may also have a component of dark matter; and they both enter the brain through the window of vomeronasal organ which is functional and has connections with the brain only during the time of ensoulment. Therefore lawgivers, philosophers, scientists and whoever related may consider the beginning of personal human life in their decisions and procedures as the 13th week of development. Before this period, embryo must be regarded as a cell cluster which is to be respected but not accorded absolute protection. The hippocampal birth theory suggests that an abortion before 13 weeks' gestation kills potential human life, whereas abortion at a later point terminates

actual human life (a person). This theory also says that society should not require funerals such a religious burial until the conception has reached 13 weeks of gestation. The hippocampal birth theory further offers a clear choice point for the ethics of embryo research: stem cell research with spare embryos produced during infertility treatment, or embryos formed specifically for research or therapeutic purposes (even by cloning), is ethically acceptable up to the point of the formation of hippocampus.

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15 Dependence of Fetal Hair and Sebaceous Glands on Fetal Adrenal Cortex and Possible Control from Epidermal Merkel Cells and Adrenal Medulla

M. Kemal Irmak

Introduction

We still do not fully understand the physiological and biological role of the fetal adrenal cortex. The purpose of the present chapter is therefore to review the literature and synthesize the current understanding of the developmental and functional biology of the fetal adrenal cortex. First, a role will be attributed to the fetal adrenal cortex in the regulation of fetal development of hairs and sebaceous glands and their common product vernix caseosa. The literature will also be discussed concerning the role of Merkel cells and adrenal medulla on the regulation of fetal adrenocortical function. As the fetal zone is principally an androgen-producing adrenal cortical zone, understanding of its regulation may provide insights into the regulation of adrenal androgen production in general.

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Development of Fetal Adrenal Cortex

Human fetal adrenal development is characterized by rapid growth, high steroidogenic activity, and a distinct morphology, including a cortical compartment known as the fetal zone. This fetal zone is a unique adrenal cortical compartment that exists only during fetal life in humans and higher primates [1–3]. Rapid growth of the human fetal adrenal cortex begins at approximately the eighth week of gestation and continues to term [4, 5]. The growth is almost entirely due to enlargement of the fetal zone and, as a consequence, the gland achieves a relative size 10- to 20-fold that of the adult adrenal by 30 weeks [1]. During midgestation, the fetal zone occupies 80–90 % of the cortical volume and produces 100–200 mg/day of the dehydroepiandrosterone sulfate (DHEA-S), which is quantitatively the principal steroid product of the fetal adrenal gland throughout gestation [6, 7]. Morphological and biochemical analyses indicate that the human fetal adrenal cortex has steroidogenic capabilities early in gestation at about 6–8 weeks [7, 8]. Estriol, as an indicative of fetal adrenal steroidogenic activity [8] can first be detected in the maternal circulation at the eighth week of gestation, indicating that DHEA-S is being produced by the fetus at this stage. At around the 12th week of gestation, estriol concentrations in the maternal circulation rapidly

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increase approximately 100-fold [9]. This increase coincides with the initiation of fetal zone hypertrophy [10]. These observations indicate that the human fetal adrenal cortex produces DHEA-S beginning at around 8–10 weeks of gestation in sufficient quantities to affect increases in maternal estriol levels. Production of DHEA-S by the fetal adrenal cortex continues for the remainder of pregnancy and during the second and third trimesters, it increases considerably such that by term the human fetal adrenal produces around 200 mg/day of DHEA-S [8].

However, the fetal adrenal, in the presence of ACTH, prolactin, and growth hormones, involutes after birth [11, 12]. Associated with these changes is a 50 % decline in the absolute weight of the adrenal during the first few postnatal weeks which is reflected by a sharp decrease in DHEA-S concentrations [12], but at the end of the third year, a new zone (the reticular zone) develops from the outermost layer of the fetal cortex [13].

Dependence of Fetal Hair and Sebaceous Glands on Adrenal Androgens

The physiological role of the fetal adrenal cortex during intrauterine life is not well understood. Early in gestation (8–10 weeks), the glands appear to be capable of DHEA-S synthesis. This event seems to be related with the differentiation of hairs and sebaceous glands. Hairs begin to develop early in the fetal period between 9 and 12 weeks [14]. The first hair that appear – **lanugo** – are fine, soft, and lightly pigmented. Lanugo is plentiful by 17–20 weeks, and covers the skin of the fetus densely and helps to hold the vernix caseosa on the skin. Lanugo is shed between the first and the fourth month postpartum, and is replaced by coarser vellus hair [15]. **Sebaceous glands** develop around the 13th to 15th gestational week as buds from the sides of developing hair follicles [16, 17] and form an oily secretion – **sebum** – that passes to the surface of the skin, where it mixes with desquamated epidermal cells to form **vernix caseosa**. Vernix caseosa is a greasy substance that covers the fetal skin and it protects the developing skin from constant exposure to amniotic fluid [18]. Human sebaceous glands attain quite a large size by the time of birth, but then shrink to comparatively small structures until the onset of adrenarche. Androgens are important in determining the type and distribution of hairs over the human body [19]. Androgens are also a prerequisite for sebaceous gland development [20] and they increase sebum secretion. Therefore, not only do androgens alter the type of hair present, but they will increase the oiliness of skin and hair. It seems that the human fetal adrenal cortex produces enough DHEA-S beginning at around 8–10 weeks of gestation to influence the growth of fetal hairs and sebaceous glands. While the fetal zone atrophies soon after birth, fetal hairs are shed and sebaceous glands shrink to small structures in concordance with the rapid decrease in adrenal androgen levels. These postnatal changes in the hair and sebaceous glands strongly support the view about the effects of fetal adrenal cortex on the development of fetal hair and sebaceous glands.

Regulation of Adrenal Androgen Secretion

After the neonatal period, until approximately 6 years of age, levels of adrenal androgens in normal children are at their lifetime minimum [21]. The zona reticularis, not perceptible in children under 6, later recapitulates the secretory pattern of the fetal zone, forming DHEA-S [22–25]. This increase takes place in girls and boys between 6 and 8 years of age, approximately 2 years before the onset of gonadal maturation [23, 26] and termed adrenarche [27]. Adrenarche is accompanied clinically by pubarche, the appearance of axillary and pubic hair [28]. After a peak of adrenal androgen production at age 20–25 [22], DHEA-S, particularly, begins a steep, continuous decline [29], reaching about 20 % of peak levels in those older than age 70. The mechanism responsible for adrenarche and the control of fetal adrenal androgen secretion have both been the subject of considerable investigation, but

remain a matter of controversy [30, 31]. Elucidation of the mechanisms has been hampered by a paucity of appropriate animal models. None of the common laboratory animals has a comparable fetal zone and the event of adrenarche occurs only in humans and higher primate species that have a long childhood preceding the advent of puberty [32–34]. It is well known that the adrenal androgens emanate chiefly from the zona reticularis of the adrenal cortex [35, 36], and hormones, such as ACTH, prolactin, gonadotropins, and estrogens, do not appear to affect adrenal androgen secretion and do not cause the adrenarche [26, 37]. Moreover, during aging in healthy people, serum levels of aldosterone, cortisol and corticosterone undergo relatively little change [29, 38, 39]; in contrast, serum concentrations and excretion of adrenal androgens decline markedly in both sexes [40, 41]. Considering the fact that there is a discrepancy in ACTH levels in plasma and androgen release in the time of adrenarche and in several other clinical situations [26, 35, 38, 42–44], adrenal androgen production seems to be regulated by a different mechanism other than ACTH.

Evidences for a Regulatory Paracrine Effect of Medullary Cells on Cortical Cells

Previous considerations support the hypothesis of a distinct adrenal androgen-stimulating mechanism which might be dependent on intra-adrenal factors that control growth and differentiation of the zona reticularis. In this respect, it was suggested that during adrenarche, it is the adrenal medulla which might influence the adrenal androgen secretion [45]. This suggestion is consistent with numerous lines of evidence which indicate that adrenal medulla exerts a paracrine control on the secretory activity of the cortex by releasing catecholamines and several regulatory peptides [46–58], suggesting that adrenomedullary function might be linked to the androgen secretion. Moreover, the dose-dependent inhibition of adrenomedullary catecholamine secretion in response to adrenal androgens [59, 60] and the presence of $ß_2$-receptors on cortical cells [61] suggest interdependence of epinephrine and DHEA secretion in the adrenal gland. Several previous findings which show the anatomical proximity and close intermingling of chromaffin and cortical cells in the adrenal [62–68], the disintegration of medullary capsule of the adrenal at adrenarche [69] and the innervation of the adrenal cortex by nerve fibers originating in the medulla [70] also suggest that sympathoadrenal hormones may play a role in the complex developmental process of adrenarche [45]. Therefore, adrenomedullary function appears to be tightly linked to adrenal cortex, explaining the variations in the adrenomedullary hormones with adrenarche [45]. Studies undertaken so far suggest a local intraadrenal peptidergic regulatory concept, and above considerations support the hypothesis that medullary cells may also be involved in the regulation of fetal cortical cell activity in a paracrine manner.

Merkel Cells and Adrenal Medulla Might Play a Role in the Fetal Adrenal Androgen Secretion

Fetal adrenal cortex seems to affect the development of fetal hair and sebaceous glands, but the mechanism that regulates fetal adrenal androgen production is a key unanswered problem in human adrenal biology. It was suggested that Merkel cells and adrenal medulla might play a role in the induction and control of fetal adrenal androgen secretion [71, 72].

The adrenal gland originates from two distinct primordial in mammals: the cortex derived from mesoderm, and the medulla derived from the ectodermal neural crest [1, 73]. What is the biological meaning of this spatial integration of two embryologically distinct organs? What are the interrelations between the two secretory cell lines? It was suggested that this fusion may be an important achievement that facilitates the adaptation to environmental changes, thus enabling better coordination of the functions of the mammals [62]. Environmental factors seem to affect the adrenal physiology. For example, adaptation of

higher animals to cold is maintained by the participation of the sympatho-adrenal system. The adaptation to cold develops successfully owing to the marked compensatory hypersecretion of epinephrine by the adrenal glands [74]. Hyperproduction of cathecolamines leads to heat production and combines with an increased reaction of the organism to epinephrine and norepinephrine [75]. That is why, in adrenalectomized animals, adaptation to cold proved impossible, and the animals died after duration of exposure to cold that was well tolerated with an intact sympatho-adrenal system [75]. Exposure to amniotic fluid during gestation is also another environmental factor from which the fetus should be protected. We can suggest therefore that long-term exposure to amniotic fluid may affect adrenal gland activity by medullary cells known to be involved in environmental adaptation.

Merkel cells also arise from stem cells of neural crest origin that migrated during the sixth embryonic week in human skin [76]. Merkel cells with a density of 1,700 per mm^2 showed cytoplasmic processes directed towards the basal lamina and extending between neighboring keratinocytes. However, these cells generally disappear at the end of gestation and innervation is necessary for the survival of Merkel cells in postnatal life [77, 78]. It has been demonstrated that Merkel cells in the developing skin transiently expressed mRNA for the serotonin transporter indicating an intact and active 5-hydroxytryptamine (5-HT) uptake system [79]. But, Merkel cells themselves are not able to synthesize 5-HT and the source of 5-HT as a substrate for the transporter in Merkel cells is the amniotic fluid, which contains 5-HT transported from the maternal circulation [80]. By expressing the 5-HT transporter, Merkel cells might have a transient role in sensing the 5-HT levels in amniotic fluid [79] and playing a trophic role for the development of fetal adrenal cortex through their endocrine secretions. Receiving or sensing the extracellular 5-HT levels may therefore allow the Merkel cells to modulate the adrenocortical function via adrenal medulla and thus to protect fetal skin from harmful effects of amniotic fluid during gestation by forming vernix caseosa. The postnatal decrease in 5-HT transporter mRNA and 5-HT uptake might reflect this physiological phenomen [79].

Conclusion

The biological meaning of the spatial integration of cortical and medullary cells seems to be a functional coordination of both epithelial cell lines as has been suggested for the interaction between parafollicular and follicular cells of the thyroid gland [81, 82]. Functional interdependence between the medullary and cortical cells explains the biological significance of their spatial integration. The colocalization of cathecholamine-secreting and steroid hormone-producing cells under a common capsule [62, 67, 68, 83–87] may, via their paracrine interactions, coordinate adaptations to stress and the environment. In this respect, fetal skin seems to be protected from an environmental stressor (contact exposure to amniotic fluid) by vernix caseosa – a product of fetal adrenal cortex, and this stimulus seems to be transferred to the adrenal gland via epidermal Merkel cells. We therefore conclude that both Merkel cells and adrenomedullary cells may facilitate the adaptation of the cortical cells to environmental changes, enabling more effective coordinated functions of the body.

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16 Theoretical Postulation of the Embryological Basis of the Virgin Birth and Role of Embryonic Stem Cells Localized Out of the Embryo

M. Kemal Irmak

Introduction

Human sex is determined at fertilization by the sex chromosome of the sperm. While X-bearing sperms form XX females, those containing a Y chromosome give rise to XY males. Y chromosome triggers the undifferentiated gonad into the path of testicular development; in its absence, the indifferent gonad develops into an ovary [1]. The differentiation of the male reproductive tract and external genitalia depends on hormones secreted by the fetal testis, whereas the fetal ovary is not required for the development of the female tract, which occurs simply in the absence of testicular secretions [2]. Primordia of female and male genital ducts are present in the early embryo of both sexes [1], and the normal development of the internal genital ducts is determined by the presence or absence of testicular tissue. In males, testicular secretions induce the development of male ducts while they lead to the regression of the female ducts [1, 2]. In females, the lack of testicular secretions allow the development of the female genital tract, while the male ducts regress due to a lack of testosterone [1].

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A Human May Have Functional Male and Female Gonads

In some persons, both ovarian and testicular tissues are present, either in the same (ovotestis) or in opposite gonads [2, 3], and they are called true hermaphrodites.

Ovulation and spermatogenesis can be observed in the gonads of these subjects [4–7]. It was reported that 35 % of true hermaphrodites have bilateral ovotestes, 38 % have one ovotestis and a contralateral ovary or testis, and 27 % have one ovary and a contralateral testis [4]. An ovotestis is a bipolar structure showing ovarian tissue in an upper pole and the testicular tissue in the lower pole [5]. Efferent ducts and vas deferens are never found together next to an ovotestis which can be explained by lower amount of testosterone [6]. While the ovarian compartment of an ovotestis shows evidence of ovulation at puberty in about 50 % of cases, spermatogenesis has never been observed in the testicular portion of an ovotestis [6, 7]. Spermatogenesis was observed in only solitary testes found in true hermaphroditism [7]. About 10 % of true hermaphrodites are derived from more than one zygote and are chimeras (chi 46,XX/46,XY) [3, 7]. In chimerism, two oocytes are fertilized by two spermatozoa, then fusion of two different zygotes into a single embryo takes place [8]. The external appearance of these subjects is extremely variable, they may be normal male and normal

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female, or ambiguous [9]. Many such cases have been reported in the literature and healthy offsprings have been observed in a few men and women [9–16].

A Left Ovary and a Right Abdominal Testis May Develop in a Human

In chimeric subjects, ovary is localized frequently on the left side of the body, while testis is found more common on the right [4]. During development of a chimera, XX cells tend to gather on the left side of the embryo while XY cells on the right [2, 17–19] which may explain the preferred locations of the gonads (left ovary and right testis). In human true hermaphrodites, regression of the male and female genital duct systems is different because of the presence of both types of the gonads. Embryonic testis has its effect mainly by local action on the adjacent ducts [20–22]. Therefore, testicular tissue may not influence the growth of the female duct on the contralateral side [23], but at least 15 % of testicular tissue is enough to inhibit the growth of the ipsilateral female duct. Moreover, the uterine and oviductal portions of the female duct differ in their distance and sensitivity to testicular secretions [21]. Thus the regression of the female duct system is variable in human true hermaphrodites [5]. The uterus was present throughout its length and normal in 10 % of cases, and present but hypoplastic in 46 %. Only one uterine horn was present in 10 % and only the uterine body was present in 14 % of subjects [5]. On the other hand, the growth of the male duct is not correlated to the amount of testicular tissue in the ipsilateral gonad [23]. One explanation for the absence of the male ducts at the right side is that the maximal response may only be triggered by a critical higher hormone level.

Transperitoneal Migration of Gamets to Contralateral Oviduct

During ovulation, estrogens increase the motility of the uterine tubes which results in a negative pressure in the oviducts [24–28]. This produces a vacuum effect which has made several pregnancies possible in subjects lacking an ipsilateral ovary by allowing the transperitoneal migration of oocyte from the contralateral gonad [29–34]. In a case of ectopic pregnancy in the left oviduct, sperms gain access to the left oviduct after entering the peritoneal cavity via the right oviduct [35]. These suggest that ipsilateral oviduct is not always essential for fertilization and negative pressure in the oviducts is much enough to pick-up the gametes on the contralateral side.

Pregnancies in Chimera

Many pregnancies were reported in chimera of 46,XX/46,XY type while a few has fathered a child [4, 36–40]. It is interesting to note that all pregnancies resulted in male fetuses [36, 41–43]. The probability of having many consecutive male newborns is unlikely to be related to chance alone. Our observations and several other studies indicate that at fertilization, warmer oocytes prefer Y-bearing sperms to give rise to a male embryo while cooler oocytes prefer X-bearing sperms to give rise to a female embryo [44–47]. Since local temperature is higher at the left ovary than the right one because of the differences in the speed of venous drainage (at the left side, slower drainage to the renal vein with a right angle; and at the right side, faster drainage to the vena cava inferior with an oblique angle result in different gonadal temperatures at the two sides of the abdomen [48]), the oocytes in those women with unilateral left ovary seem to prefer Y-bearing sperms resulting in pregnancies with male embryo. Cooler temperature at the right side also seems to allow the testis to be functional at the right side of the abdominal cavity.

Self-Fertilization

Self-fertilization was reported in many flowering plants, in a kind of fish and in a case of rabbit [49–52]. They have both eggs and sperms in their body and at fertilization, one sperm cell fuses with oocyte to form an embryo. The mangrove

killifish which can reproduce by self-fertilization [51] has bilobed gonad consisting mainly of ovarian tissue with small amount of testicular tissue that simultaneously produces eggs and sperm [52]. It is interesting to note that a true hermaphrodite rabbit with two functional ovaries and two testes was housed in isolation and became pregnant and delivered seven healthy young of both sexes [50]. Is self-fertilization possible for a human? The answer is yes, but it seems to occur once in the history.

Scenario for Mary to Give Birth to Jesus without a Father

Virgin Mary is a chimera of 46,XX/46,XY type resulting from the fusion of two embryos of different sex types and both ovarian and testicular tissues develop in Her body as seen in a beautiful plant [53]. In another word, She develops from the fusion of dizygotic male and female twins and She had both reproductive cell types (oocytes and sperms) in Her body. Since XX cells tend to gather on the left side while XY cells on the right, an ovary develops on the left side with a patent oviduct and a testis develops at the right side of the abdominal cavity with no duct [54]. Her intact female reproductive tract on the left side indicates that contralateral testicular secretion is not adequate to induce complete duct regression during development on the left. Female genital duct regression on the right side is however mediated by the secretions from the ipsilateral testis, but testosterone secreted from right testis does not prevent the regression of the male duct due to a lack of response to it or inadequate hormone levels at the site. Therefore, no duct is present next to the testis at the right side, and tubules of the testis have an open access to the abdominal cavity allowing the sperms to be picked-up by the contralateral oviduct. Both gonads are functional and produce oocyte and spermatozoa respectively after puberty. At the time of ovulation, estrogens increase the motility of the oviduct on the left side which results in a negative pressure in the tube and oocyte and sperms are picked-up into the tube with the help of this vacuum effect, taking both gametes into the fertilization site in the oviduct. Because of the higher temperature of the oocyte derived from the left ovary, fertilization occurs with a Y-bearing sperm to give rise to a XY male embryo which becomes miraculously Jesus [55].

Embryonic Stem Cells Localized out of the Embryo

In mammals, all cells produced by the first few divisions of the fertilized egg are totipotent – that is, they all have the potential to give rise to an embryo with the placenta [56]. Each cell (blastomere) is equipotent up to the eight-cell stage (Fig. 16.1a), after which there is a gradual restriction of the developmental potency of the cells [57]. The differentiation of the inner cell mass and the trophoblast lineage is the first restriction event in embryogenesis. The inside blastomeres are made up of a population of pluripotent cells and are called inner cell mass which give rise to the embryo, whereas the outside cells give rise to the trophoblast (Fig. 16.1b), which is the future placenta [58]. After implantation takes place, the inner cell mass develops to a simple epithelium called epiblast (Fig. 16.1c). Epiblast cells are still pluripotent after the implantation [59].

While all somatic cells are destined for death once they start differentiation, germ cells are the only cells which can give rise to successive generations. Primordial germ cells, the earliest recognizable precursors of germ cells are recognizable at a surprisingly early stage in development [60]. They arise from a pluripotent population of cells in the epiblast (Fig. 16.1c) [61] and then take up temporary residence in extraembryonic tissues before returning to the body of the embryo proper [62]. Primordial germ cells actively migrate into the proximal allantois, and directly into the adjacent embryonic endoderm [63]. They spend the early stages of development within these extraembryonic tissues as a small cluster of cells [64]. The allantois arises early in the third week as an endodermal outgrowth from the dorsocaudal part of the yolk sac into the connecting stalk. The main function of

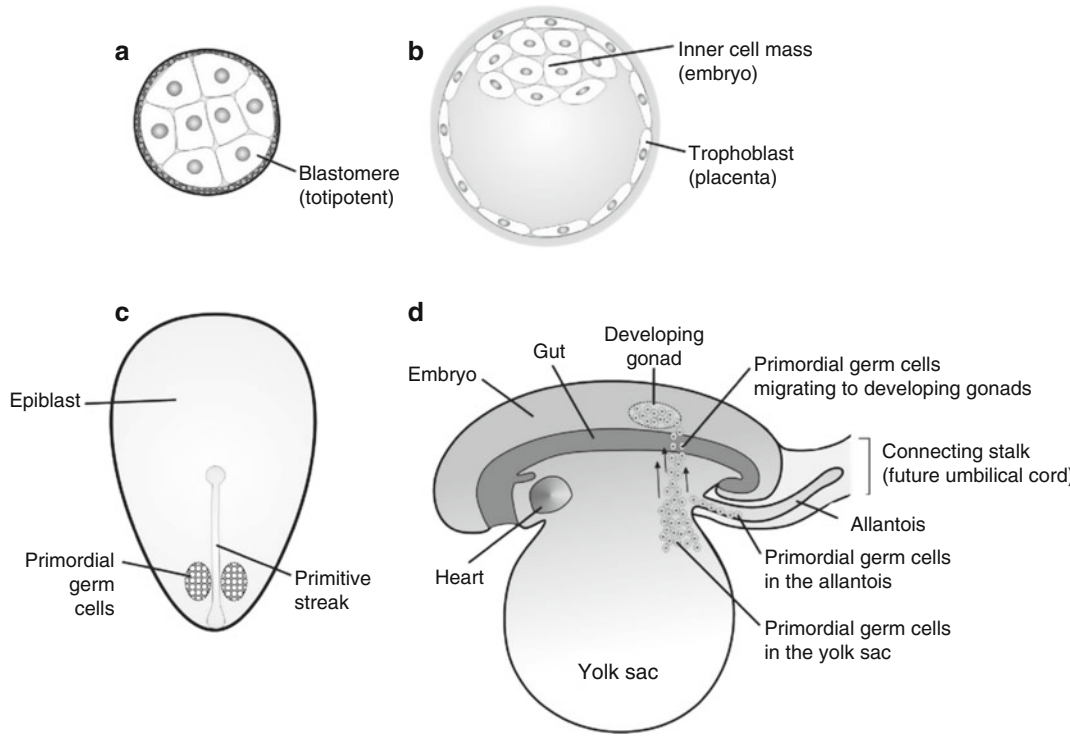

Fig. 16.1 Developmental fate of a group of embryonic stem cells in the early developmental stages: (**a**) All cells up to the eight-cell stage after the first few divisions of the fertilized egg are totipotent – that is, all cells (blastomeres) have the potential to give rise to an embryo with the placenta. (**b**) The differentiation of the inner cell mass which gives rise to the embryo and the trophoblast lineage which is the future placenta is the first restriction event in embryogenesis. (**c**) Primordial germ cells arise from a pluripotent population of cells in the epiblast. (**d**) Pluripotent primordial germ cells that enter the allantois remain in the allantois and do not return to the embryo whereas other primordial germ cells migrate dorsocranially in the embryo to reach the developing gonads

the allantois is to fuse with the chorion and vascularize, thereby forming the umbilical component of the placenta [62]. Evidence suggests that bone morphogenetic protein 4 secreted by cells lying outside the embryo proper is required for the formation of both primordial germ cells and allantois [65]. These cells divide and mature, turning off the expression of a number of somatic cell genes and turning on the expression of genes involved in maintaining the special character of the germ cells and allantois [56].

Primordial germ cells are found at the base of the allantois and in the adjacent endoderm of yolk sac in the early developmental stages [66]. All of these cells do not migrate into the embryo, only a specific subpopulation does. The population of primordial germ cells that enter the allantois contribute only to the allantois, and do not return to the embryo [62, 67, 68], whereas other primordial germ cells migrate dorsocranially in the embryo to reach the developing gonads (Fig. 16.1d) [69]. The involution of allantois results in the formation of the urachus and medial umbilical ligament localized between the urinary bladder and the navel [60]. Allantoid remains and primordial germ cells may still be present inside the ligament all the way to adulthood; which can develop into cysts, diverticula and neoplasms [70].

The base of the allantois which contains the future germ line is the only allantoic region to exhibit pluripotency, colonizing several derivatives of all three primary germ layers [68]. These cells retain an unrestricted developmental potential and can give rise to all the tissues and cell types in the body, including germ cells [56]. Thus,

in a suitable environment, they can produce any of the cell types of the body, although not the extra-embryonic cells that go on to form structures such as the placenta; for this reason, they are said to be pluripotent, rather than totipotent [57]. Cells with this property are called embryonic stem cells [56]. By not migrating into the embryo and staying in the allantois arrests the normal program of change of their character with time and so enables these stem cells to live without differentiating [56]. It seems that in the early developmental stages, a group of pluripotent embryonic stem cells each of which can give rise to an embryo are set aside out of the embryo for a possible use, may be a rebirth, in the future [71].

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Aneuploidy in Human Preimplantation Embryos

17

Joseph P.M. Geraedts

Introduction

Even when conditions are optimal, the chance of a clinically recognized pregnancy occurring in a given menstrual cycle is much less than half. Besides failure of conception pre-clinical pregnancy loss is a major cause for the low fecundity observed in humans. Chromosome abnormalities are the predominant cause of this clinical problem, both in natural conceptions as well as after assisted reproduction [21]. About 42 % of all oocytes exposed to natural fertilization survive until after implantation, and only 31 % will lead to a live birth (Fig. 17.1).

The number of abnormal zygotes cannot simply be calculated on the basis of the aneuploidy rates of gametes but may result from abnormal fertilisation. Furthermore mitotic abnormalities during cleavage division give rise to mosaic chromosome abnormalities of the embryos.

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Lessons from Cytogenetics

The frequency of chromosome abnormalities in oocytes, zygotes and early stages of embryo development have been studied since the early 1980s of last century. After the introduction of fertilisation in the IVF laboratory as a means of infertility treatment it has become possible to study human gametes and the early stages of embryonic development. Due to legal restrictions in most countries it is not allowed to create embryos for study purposes and the superfluous embryos which originate within the context of infertility treatment do not reflect the normal situation. However, it is possible to obtain normal oocytes from couples with male factor infertility and visa versa. Furthermore, the introduction of molecular methods has allowed more unselected studies to be carried out. Fluorescence In Situ Hybridization (FISH) has first provided the opportunity to analyse the chromosomal content of the earliest conception products [28]. It allows the detection of aneuploidy in single cells. However, only a limited number of chromosomes can be studied using this method. Furthermore, the estimation of hypohaploidy (loss of chromosomes) is more problematic using FISH.

Therefore the more recent introduction of techniques that allow the simultaneous study of all chromosomes have contributed most. Metaphase comparative genomic hybridisation (mCGH) was

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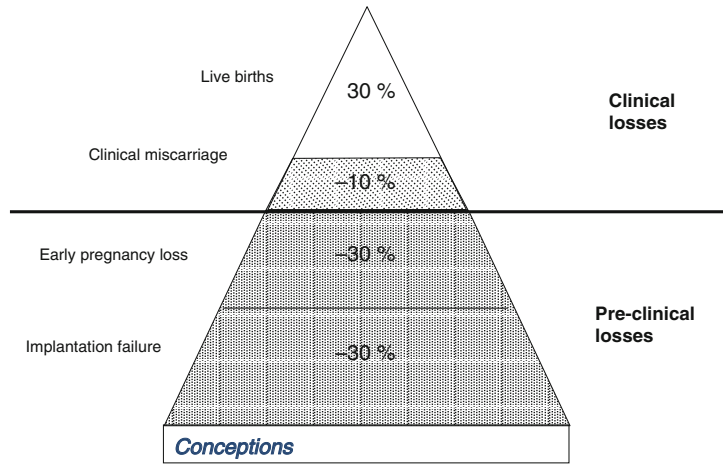

Fig. 17.1 The pregnancy loss iceburg: an overview of the outcome of spontaneous human pregnancy. Seventy percent of conceptions are lost prior to live birth. The majority of these losses occur prior to the time of the missed menstrual period, and are not revealed (Modified from Chard [5] with permission from Elsevier. Modified by [21]. By permission of Oxford University Press)

originally developed for cytogenetic analysis of solid tumors [17]. At the beginning of this century it has been introduced for the study of preimplantation embryos [37, 38]. The principle of this method is that control and study DNA are simultaneously hybridised onto a normal metaphase. On the basis of a shift in the ratio of two colours quantitative fluorescence microscopy reveals chromosomes (in case of aneuploidy) and chromosomal regions (in case of unbalanced structural rearrangements) which are lost or amplified. Based on the same principle as metaphase CGH, array CGH differs in that genomic clones from selected regions of the genome are spotted on a slide, replacing normal 'control' metaphase cells as the target DNA [19]. Since then various other technologies have been developed that allow copy number analysis of all 23 pairs of chromosomes, 22 autosomes, and the sex chromosomes, or "24-chromosome" copy number analysis in single or small numbers of cells [13].

Aneuploidy in Oocytes

Since the first report on the study of meiosis in human oocytes [39], it has become known that aneuploidy frequently arises during female meiosis and that it is closely associated with advancing maternal age. The relationship between increasing maternal age and trisomy is well recognized [16]. The risk of trisomy in a clinically recognized pregnancy rises from about 2–3 % for women in their 20s to an astounding 30 % or more for women in their 40s [16].

Published reports of full karyotype analysis on the first polar body, confirmed by the results of MII oocyte analysis, indicate that there is a low aneuploidy rate (3 % in one study) in oocytes of young fertile women [8]. To shed further light on the nature of aneuploidy in human oocytes, CGH was also used to provide a detailed cytogenetic analysis of first and second polar bodies biopsied from the fertilized oocytes of women with an average age of more than 40 years [7, 9]. The total oocyte abnormality rate was 70 % and MII anomalies predominated over MI (50 % aneuploidy rate versus 40.3 %).

In a large-scale series of over 20,000 oocytes, aneuploidies were detected in polar bodies using FISH for chromosomes 13, 16, 18, 21 and 22. Almost every second oocyte (46.8 %) was found to be abnormal, with a predominance of errors involving chromatid loss, predicting predominance of trisomies (53 %) over monosomies (26 %) in the resulting embryos [18]. Of the detected anomalies in oocytes, 40 % were complex.

Therefore it is clear that both female meiotic divisions and all chromosomes can be, and frequently are, affected by errors in their segregation. In general, oocyte chromosome abnormalities increase dramatically in women over the age of 40 years. This steep increase in

the presence of chromosome errors may reflect adverse intrinsic (oocyte) or extrinsic (ovarian or hormonal) effects related to advancing maternal age. However, it is noteworthy that the vast majority of existing data on maternally derived aneuploidy come from oocytes generated via ovarian stimulation, and it remains possible that oocytes from natural ovulatory cycles may exhibit some differences [7, 9].

There seems to be no effect of maternal age on triploidy and tetraploidy. These abnormalities originate at fertilisation and during preimplantation development respectively. This confirms that maternal age is a factor, which only influences meiosis.

The cause of non-disjunction in oocytes of older women is largely unknown. The classic textbook mechanism for aneuploidy arising in meiosis is non-disjunction of whole chromosomes or sister chromatids in the first (meiosis I) and second meiotic divisions (meiosis II), respectively [10]. Premature centromere division during meiosis-I has been suggested as an alternative mechanism for trisomy formation [1, 2]. This could be studied for the first time at a large scale in a series of women having aneuploidy testing by array CGH mainly because of advanced maternal age (mean age 40 years). Nearly three quarters (72 %) had one or more aneuploidies in either one or both polar bodies, which was confirmed in almost all corresponding fertilised oocytes [12]. By examining the copy number changes resulting from these meiotic errors, in the three products of female meiosis, that is, both polar bodies and the corresponding zygote, it was possible to distinguish non-disjunction from premature predivision of sister chromatids as the cause of aneuploidy in the corresponding zygotes. The results of this analysis demonstrated that almost all meiosis I errors for all chromosomes are caused by premature predivision of sister chromatids. As would be expected by the random segregation of single chromatids at meiosis II to either the PB2 or the zygote, however, a significant proportion of meiosis I errors did not result in aneuploidy in the zygote. Overall, therefore, there were more meiosis II-derived maternal aneuploidies in the zygote. Furthermore, over half of the zygotes examined had multiple aneuploidies [14].

Aneuploidy in Spermatozoa

Using FISH the majority of chromosomes have been studied in spermatozoa. However specific data are lacking for chromosomes 5, 10, 11, 14, 17 and 19. The majority of authors report on chromosomes 13, 18 and 21 and the sex chromosomes, thus, the estimates of aneuploidy for these chromosomes are the most reliable. It has been found that disomy frequencies differ among chromosomes. For autosomes, disomy is in the order of 0.1 % but may range from 0.03 (chromosome 8) to 0.47 (chromosome 22). The great majority of authors find that chromosome 21 (0.17 %) has a significantly elevated frequency of disomy, and that the sex chromosomes have the highest frequency (0.27 %), although these findings are not universal. The lower estimate of total aneuploidy in FISH studies is 4.5 % (2× disomy of 2.26 %) [33]. The variations observed between studies can be attributed to technical factors such as decondensation and denaturation of the DNA in the specimen and distortion of the morphology after pretreatment. Besides technical factors explaining a proportion of the variation observed, it should not be forgotten that differences might be observed between patients. It has been shown that the percentage of numerical abnormalities is significantly increased in a group of patients with abnormal semen characteristics [27, 35]. Finally it has been observed that sperm of non-obstructive azoospermic men had a higher incidence of chromosome abnormalities, of which sex chromosome abnormality was the most predominant [24]. A higher incidence of numerical chromosome abnormalities in sperm has also been detected in ICSI zygotes [20]. This is confirmed by the limited available data on ICSI foetal karyotypes which reveal that, in comparison with a general neonatal population, there is: (i) a slight but significant increase in de-novo sex chromosomal aneuploidy (0.6 % instead of 0.2 %) and structural autosomal abnormalities (0.4 % instead of 0.07 %) [34].

Abnormalities in Preimplantation Embryos

The reported rate of abnormal fertilisation observed during IVF varies from 2 % to about 9 %. About 20 h after insemination, the zygotes are studied for the presence of pronuclei. The two main categories of abnormalities are parthenogenetic activation (one pronucleus) and triploidy (three pronuclei). Tripronuclear zygotes result in most cases from dispermy [29].

Cytogenetic studies of preimplantation embryos carried out using classical techniques have reported rates of abnormalities which varied from 23 % to 90 % [21].

The development of FISH technology first enabled arrested human embryos to be studied. These studies revealed mostly mosaic chromosome complements [6, 15]. Also in dividing preimplantation embryos chromosomal mosaicism has been shown to be a normal feature of both morphologically abnormal [4, 25, 26] as well as normal embryos. Chromosomal mosaicism might be one of the reasons why the application of preimplantation aneuploidy screening to ensure transfer of euploid and improvements of live-birth rates from IVF has not worked using FISH [11]. The first studies applying the (metaphase) comparative genome hybridisation technique reported an identical result of 3 out of 12 preimplantation embryos consisting of only normal cells [36, 37]. In 2013 Mertzanidou et al. [22] used aCGH to study all blastomeres from 14 good-quality human preimplantation embryos from <35 years old) IVF patients. Only four of these were uniformly diploid, while the remaining ten were mosaic. None of the embryos had the same aneuploidy pattern in all cells. Of the 70 analysed blastomeres, 55.7 % were diploid and 44.3 % had chromosomal abnormalities, while 29 % of the abnormal cells carried structural aberrations.

It has been reported that during preimplantation development, a high prevalence of mosaicism persists to the blastocyst stage [3, 30]. However, the percentage of abnormal cells per embryo was 16 %, which is less than that of cleavage stage embryos. At the blastocyst stage aneuploidies were found to be significantly less common among embryos of optimal morphological quality, while such abnormalities were overrepresented amongst embryos considered to be of poor morphology. However, many blastocysts affected by forms of aneuploidy with the greatest capacity to produce clinical pregnancies, such as trisomy 21, were indistinguishable from euploid embryos [9]. Even though there is a strong selection against chromosomally abnormal embryos, extended culture to day 5 or 6 cannot be used as a reliable tool to select against clinically relevant chromosome abnormalities such as trisomies [31].

Already during preimplantation development the potential of human embryos depends on their chromosomal constitution. The majority of all normal embryos reach the blastocyst stage, while this is the case in only a minority of chromosomally abnormal embryos [31]. Since trisomies and triploidies are much more viable than monosomies and more complex aneuploidies, human miscarriage in the first trimester mainly results from embryos with extra chromosomes [32]. Recently a meta-analysis was performed to obtain a more precise evaluation of the risk of embryonic chromosomal abnormalities in first-trimester miscarriage after ART in which a total of 15 studies were included. No statistical difference was found in risk of chromosomally abnormal miscarriage compared with natural conception and the different types of ART utilized, whereas the risk of foetal aneuploidy significantly increased with maternal age ≥35 [23].

Conclusion

To a large extend, the low fecundity of humans can be explained by preimplantation embryonic loss as a result of the occurrence of chromosomal abnormalities in the human conceptus. They can originate during gamete formation, at fertilization and during various stages of early embryo development. As a result of the latter, the majority of cleavage stage embryos created by IVF are chromosomal mosaics. The contribution of abnormalities via sperm is much less than via the oocyte. Aneuploidy of meiotic origin in older women mainly results from premature centromere

division. In young women the vast majority of chromosomally abnormal embryos are caused by post-zygotic mitotic errors.

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18 The Potential Impact of Maternal Milk Consumption During Pregnancy on mTORC1-Driven Fetal Growth

Bodo C. Melnik

Abbreviations

4E-BP-1	Eukaryotic translation initiation factor 4E-binding protein 1
AAs	Amino acids
AGA	Appropriate for gestational age
AKT	V-AKT murine thymoma viral oncogene homolog (protein kinase B)
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
BCAA	Branched-chain amino acid
BM	Basal membrane
BMI	Body mass index
EIF4A	Eukaryotic translation initiation factor 4A
EIF4E	Eukaryotic translation initiation factor 4E
ERK	Mitogen-activated protein kinase kinase 4
FFA	Free fatty acid
FGF21	Fibroblast growth factor 21
GDH	Glutamate dehydrogenase
GDM	Gestational diabetes mellitus
GF	Growth factors
GH	Growth hormone
GHR	Growth hormone receptor
GIP	Glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GLP-1	Glucagon-like peptide-1
GRB2	Growth factor receptor-bound protein 2
HPL	Human placental lactogen
IGF-1	Insulin-like growth factor-1
IGF1R	Insulin-like growth factor-1 receptor
IR	Insulin receptor
IRS	Insulin receptor substrate
JAK	Janus-activated kinase
LAT	L-type amino acid transporter
Leu	Leucine
LGA	Large for gestational age
MEK	Mitogen-activated protein kinase kinase 1
MiR	Micro-ribonucleic acid
mTORC1	Mechanistic (mammalian) target of rapamycin complex 1
MVM	Microvillous plasma membrane
PDCD4	Programmed cell death 4
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PGH	Placental growth hormone
PI3K	Phosphoinositide-3 kinase
PRLR	Prolactin receptor
PTEN	Phosphatase and tensin homolog
RAF	V-RAF-1 murine leukemia viral oncogene homolog
RAS	V-HA-RAS rat sarcoma viral oncogene homolog

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RHEB	RAS homolog enriched in brain
S6K1	Ribosomal protein S6 kinase 70-kD kinase 1
SOCS	Suppressor of cytokine signaling
SPRY2	Sprouty2
Trp	Tryptophan
TSC2	Tuberin

Introduction

Continued consumption of cow's milk and dairy products during adolescence, adulthood and pregnancy is an evolutionarily novel behavior that may have long-term adverse effects on human health [1]. Milk is a highly specialized, complex signaling system developed by mammalian evolution to promote mTORC1-driven postnatal growth [2]. Milk is not designed by nature to promote prenatal fetal growth. The consumption of milk of another mammal during pregnancy is an exceptional behavior of Neolithic humans and is not observed in any other mammal on the planet. Milk is the only and sufficient nutrient system that allows adequate postnatal growth and anabolism of the newborn milk recipient. On the molecular level, cell growth, cell proliferation, nucleotide-, protein- and lipid synthesis, and other anabolic metabolic processes, and inhibition of autophagy are all mediated by the nutrient-sensitive kinase *mechanistic target of rapamycin complex 1* (mTORC1) [3–12].

After a short introduction into the major regulatory pathways activating mTORC1, mTORC1-activating nutritional and hormonal signals induced by persistent cow's milk and dairy protein consumption will be presented. After that, the effect or increased insulin/IGF-1 and amino acid signaling induced by cow's milk consumption on placental growth and placental nutrient transfer will be dissected. Finally, recent epidemiological evidence will be presented that confirms the association between increased maternal weight gain during pregnancy, increased placental weight, enhanced fetal growth and increased birth weight with milk consumption during pregnancy. In contrast to cow's milk consumption, inhibition of placental mTORC1 and insulin/IGF-1 signaling resulted in downregulation of placental nutrient transporters and link maternal undernutrition to mTORC1-mediated restriction of fetal growth [13].

mTORC1: The Nutrient-Sensitive Kinase Orchestrating Cell Growth

The nutrient- and growth factor-sensitive kinase mTORC1 is the cell's central regulator of growth and proliferation [3–12]. mTORC1 activates nucleotide, protein and lipids synthesis under conditions of nutrient availability. mTORC1 is activated at the lysosomal membrane. Two major signaling pathways are of pivotal importance for mTORC1 activation: (1) the growth factor-PI3K-AKT pathway that activates the GTPase RHEB and (2) the translocation of mTORC1 to the lysosomal site of activation via amino acid-mediated activation of the small GTPase RAGA and RAGB.

mTOR is a multi-domain protein of approximately 300 kDa exhibiting a serine/threonine protein kinase domain at its C-terminus related to phosphoinositol-3-kinases (PI3Ks). In mammalian cells two functionally different mTOR complexes exist: mTORC1 and mTORC2, respectively [3–7]. Among other functional proteins, mTORC1 contains the important partner protein RAPTOR, which interacts with substrates for mTORC1-mediated phosphorylation. mTORC1 controls the G_1/S transition and G_2/M progression of the cell cycle [14]. In contrast to mTORC2, which contains the partner protein RICTOR, only mTORC1 plays a special role in sensing cellular nutrients such as glucose, amino acids, palmitic acid, energy (ATP) levels, and cell stress (ROS) all important for the regulation of cell growth and proliferation.

Insulin/IGF-1 Signaling Activates mTORC1

Growth factor signals such as insulin and IGF-1 are integrated by the tuberous sclerosus protein TSC1 (hamartin) and TSC2 (tuberin) that regulate

RHEB (RAS-homolog enriched in brain), one essential activator of mTORC1 [15–20]. In its GTP-bound form, RHEB directly activates mTORC1. The RHEB-specific GTPase-activating protein (GAP) is the TSC2 protein, which functions as a heterotrimer with its binding partners TSC1 and TBC1D7 [20]. Growth factor signaling via TSC2 phosphorylation reduces the inhibitory function of the TSC1/TSC2/TBC1D7 complex towards RHEB which results in activation of RHEB and finally of mTORC1.

Amino Acid-Mediated Activation of mTORC1

Amino acids, especially leucine, glutamine and arginine, play a most important role for the activation of mTORC1 [5–11]. Amino acids activate mTORC1 even in the absence of insulin but not vice versa [9, 21, 22]. The activation of mTORC1 depends on two major pathways: (1) by the upstream activation of RHEB by signals derived from growth factors and (2) by the amino acid-dependent translocation of inactive mTORC1 to active RHEB localized in lysosome compartments [23–25]. Insulin and IGF-1 signaling, via activated AKT phosphorylate TSC2 and thereby suppress the inhibitory function of the TSC1/TSC2/TBC1D7 complex towards RHEB. The TSC complex associates with the lysosome in a RHEB-dependent manner, and its dissociation in response to insulin requires AKT-mediated TSC2 phosphorylation. These recent findings provide a unifying mechanism by which independent pathways affecting the spatial recruitment of mTORC1 and the TSC complex to RHEB at the lysosomal surface serve to integrate diverse growth signals [26]. The inhibition of either TSC1 or TSC2 leads to activation of RHEB and ultimately of mTORC1 [19, 26, 27].

Amino acid uptake into the cell is crucial for mTORC1 signaling. Nicklin et al. [28] suggested that cellular export of glutamine is required for cellular leucine uptake and subsequent leucine-mediated mTORC1 activation. Intracellular glutamine is required for preloading the SLC7A5/SLC3A2 bidirectional amino acid transporter, which drives the efflux of glutamine and influx of leucine for leucine-mediated mTORC1 activation [28, 29]. Remarkably, in response to amino acid depletion, mTORC1 activity is rapidly abolished [30], and amino acid starvation even impairs binding of mTORC1 to RHEB [31].

Amino acids play a pivotal role in the translocation of inactive mTORC1 to lysosomal compartments enriched in activated RHEB [23, 24]. The spatial regulation of inactive mTORC1 by amino acids is mediated by an active RAG heterodimer and is of crucial importance for amino acid sensing and activation of mTORC1 [32]. The pentameric Ragulator complex acts as a scaffold for the RAG GTPases and mTORC1 at the lysosomal membrane. According to the recent opinion, RHEB and RAGs come together at the lysosome to activate mTORC1 [26, 33] (Fig. 18.1).

Amino acid accumulation in the lysosomal lumen generates an activating signal that is transmitted in a vacuolar H^+-ATPase (v-ATPase)-dependent fashion to activate the guanine nucleotide exchange factor (GEF) activity of Ragulator towards RAGA. Upon RAGA-GTP loading, mTORC1 is recruited to the lysosomal surface where it interacts with RHEB and becomes activated [32]. Thus, mTORC1 integrates insulin, IGF-1, energy-derived signals to RHEB but in parallel requires sufficient amino acid signals for maximal mTORC1 activity [34]. Recent evidence has been provided that proton-assisted amino acid transporters (PATs) localized on late endosomes and lysosomes (LEL) interact with RAGs and are required for mTORC1 activation [35]. PAT1 (SLC36A1) is expressed at the luminal surface of the small intestine and is commonly found in lysosomes of many cell types [36]. The v-ATPase interacts with the activated RAG/Ragulator complex to control amino acid-dependent mTORC1 activation, which is regulated by the rapid accumulation of extracellular amino acids in LELs [37, 38]. Thus, in response to amino acids these molecules form a signaling complex that has been called the ‘nutrisome’ [35, 37]. Cycling of protons through this nutrisomal engine induces conformational changes that may activate mTORC1. Importantly, signaling from the insulin receptor

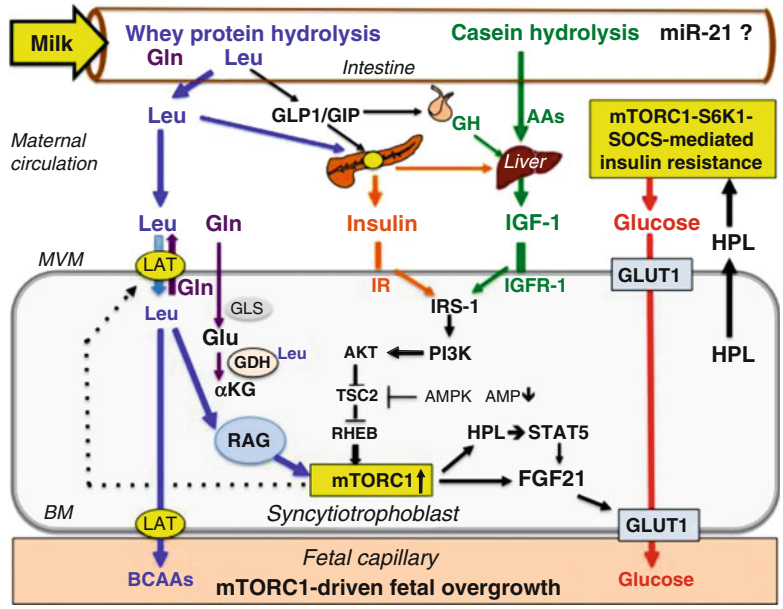

Fig. 18.1 Synopsis of milk-modified mTORC1-signaling of the human syncytiotrophoblast. Leucine (*Leu*) and glutamine (*Gln*) derived from whey protein hydrolysis increase postprandial serum insulin levels. Milk protein consumption increases serum levels of IGF-1. Leucine, insulin and IGF-1 stimulate mTORC1 of the syncytiotrophoblast and placenta growth in general. Milk consumption is associated with placental growth. Placental weight is related to increased serum levels of human placental lactogen (*HPL*). HPL via STAT5/SOCS signaling as well as increased maternal mTORC1/S6K1 signaling induce insulin resistance increasing maternal serum levels of insulin and glucose. Increased trophoblast mTORC1 activity and HPL stimulate placental expression of FGF21 that upregulates GLUT1 expression. Thus milk consumption promotes exaggerated glucose transfer to the fetus. As trophoblast mTORC1 activity controls the expression of L-type amino acid transporters (*LAT*) (*dotted line*), milk not only provides abundant leucine but also stimulates trophoblast-mediated leucine transfer to the fetus. Both glucose and leucine are pivotal stimuli required for mTORC1-driven fetal growth. Milk, a signaling system designed for postnatal growth, apparently interferes with prenatal growth trajectories when "abused" by humans during pregnancy

and subsequent activation of the PI3K/AKT/RHEB cascade promotes shuttling of PATs from the cell surface to LEL membranes, hence increasing PAT-dependent mTORC1 activation and cell growth [35, 37]. In addition, the accumulation of amino acids in the LEL lumen presumably involves transport into intracellular endosomal compartments via currently unknown amino acid transporters (AATs) or potentially via endocytosis. Cytoplasmic leucine, which is brought into cells via the heterodimeric amino acid transporter CD98 [39], has been shown to play a key role in activating mTORC1 in some cultured cells and may be important in this process. Influx of leucine or other amino acids into the LEL system may ultimately allow the amino acid substrates of PAT1 to accumulate in the LELs through amino acid exchange mechanisms, leading to PAT1-mediated activation of the nutrisome [35].

Leucyl-tRNA synthetase (LeuRS) acts as a cytosolic amino acid sensor [40, 41]. LeuRS also plays a critical role in amino acid-induced mTORC1 activation by sensing intracellular leucine concentration and initiating mTORC1 activation by binding to and activating RAG GTPase [40, 41]. LeuRS acts as a GTPase-activating protein (GAP) for RAGD, enhancing the GTP-bound form of RAGA/B crucial for amino acid-mediated mTORC1 activation [41].

The Glutaminolysis Pathway Activates mTORC1

Duran et al. [42] suggested that leucine stimulates mTORC1 indirectly through its effects on glutaminolysis. Glutamine in combination with leucine increased GTP charging of exogeneously

expressed RAGB, promoting mTORC1 activation by enhancing glutaminolysis and the production of α-ketoglutarate (α-KG) [42]. In contrast, the eIF2α (eukaryotic initiation factor 2α) kinase GCN2 (general amino acid control-nonderepressible 2) senses the absence of one or more amino acids by virtue of direct binding to uncharged cognate tRNAs [43].

Palmitate Activates mTORC1

It has recently been demonstrated that the saturated C16:0 fatty acid palmitate activates mTORC1 [44]. Palmitate activated mTORC1 by enhancing recruitment of mTOR onto lysosomal membranes [44]. Notably, the Ragulator complex component p18 is anchored to the lysosomal membrane via palmitoylation, however palmitate did not alter localization of either p18 or RAGC proteins [44]. Thus, the mechanism of palmitate-induced mTORC1 activation is yet unknown.

Glucose Activates mTORC1

Glucose is the major carbohydrate and energy source of the cell that via glycolysis is converted into pyruvate. The first step of glycolysis is the phosphorylation of glucose by hexokinase-II to form glucose 6-phosphate. The free energy released in the process of glycolysis and subsequent downstream oxidation of citrate in the tricarboxylic acid cycle is used to form the high energy compound adenosine triphosphate (ATP). High cellular ATP levels result in low levels of adenosine monophosphate (AMP). In states of starvation or restriction of glucose increased levels of AMP activate the energy-sensing enzyme AMP-activated protein kinase (AMPK). Liver kinase B1 (LKB1) and AMPK are critical regulators of mTORC1 [45, 46]. The serine/threonine kinase LKB1 represents the major kinase phosphorylating the AMPK activation loop (α-subunit of AMPK) under conditions of energy stress [47, 48]. Thus, AMPK plays a key role in energy-dependent regulation of mTORC1. AMPK is activated during energy-deficient conditions, when AMP levels rise. AMPK phosphorylates TSC2 and RAPTOR, thereby suppressing mTORC1 activity [15]. Activation of AMPK inhibits the mTORC1 pathway, activates the autophagy initiating kinase ULK-1 [49, 50]. In response to glucose deprivation, hexokinase-II, which catalyzes the first step of glycolysis, binds to mTORC1 through its TOS motif, decreasing mTORC1 activity [51, 52]. Thus, there exists a multilayer regulation of glucose-mediated mTORC1 activation [53].

Cow's Milk Consumption Increases mTORC1 Signaling

As outlined above, mTORC1 is activated by four major pathways: (1) growth factor signaling via insulin and IGF-1, (2) amino acid signaling predominantly via leucine, (3) glutaminolysis-mediated signaling, and (4) saturated fatty acid-derived signals primarily via palmitate. It will be shown that persistent cow's milk consumption in humans stimulates all four signaling pathways activating mTORC1 of the cell's of the milk consumer (Fig. 18.1).

Milk Induces Hyperinsulinemia

Milk is a materno-neonatal vector system that stimulates insulin production of pancreatic β-cells of the milk recipient, physiologically of the species-specific newborn mammal until weaning [2]. The *insulinemic index* of whole cow's milk (148 ± 14) and skim milk (140 ± 13) is much higher than the glycemic indices of whole milk (42 ± 5) and skim milk (37 ± 9), respectively [54, 55]. Fast hydrolysis and immediate intestinal absorption of insulinotropic amino acids of the whey protein fraction of cow's milk [56, 57] raises insulin levels of much higher magnitudes than intestinal digestion of structural proteins such as beef (*insulinemic index*: 51). A recent study in healthy humans confirmed that maximal plasma amino acid increase occurs within the first hour after whey protein ingestion [58]. Whey proteins, the animal proteins that contain the highest amounts insulinotropic branched-chain

amino acids (BCAAs), are the proteins that exhibit fastest hydrolysis [59]. The major insulinotropic protein fraction of cow's milk is the whey protein fraction in comparison to the casein fraction that preferentially elevates serum IGF-1 levels [60]. Furthermore, whey-derived leucine and other whey-derived amino acids stimulate the incretin secretion of enteroendocrine K- and L-cells [61, 62]. Milk is a unique carrier of essential BCAAs. Whey protein contains the highest amount of leucine (14 %), followed by total milk proteins (10 % leucine), and casein (10 %) compared to egg protein (8.5 %), meat protein (8.0 %), soy protein (8.0 %), and wheat protein (7.0 %), respectively [63]. Enteroendocrine K- and L- cells sense whey protein-derived amino acids, especially leucine, and respond by release of the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [64–66] (Fig. 18.1). In the neonatal breastfeeding period glucose-dependent insulinotropic polypeptide (GIP) signals not primarily in response to glucose but predominantly in response to whey proteins, thus operates as a whey-dependent insulinotropic polypeptide ("WIP").

Apart from the incretin induction of whey proteins, whey-derived amino acids, predominantly leucine and glutamine, exert direct inulinotropic effects on pancreatic β-cells [67–69]. It is thus fully comprehensible, that milk protein consumption in comparison to meat protein intake results in hyperinsulinemia as demonstrated in healthy 8-year old boys differentially fed with equal amounts of either milk or meat protein [70].

In obesity, an unfavorable condition promoting fetal overgrowth, plasma leucine levels are significantly elevated [71]. It has been demonstrated in obese children that additional supply of leucine resulted in excessive hyperinsulinemia [72]. Thus, there appears to be a synergism of hyperinsulinemia mediated by milk-derived leucine intake and obesity-associated hyperleucinemia. Both metabolic deviations may be unfavorable during pregnancy and may adversely affect placental insulin-dependent nutrient transfer to the fetus as discussed later.

Milk Increases Serum IGF-1 Levels

A meta-analysis confirmed that continued milk consumption increases serum levels of the growth hormone insulin-like growth factor-1 (IGF-1) [73]. The *European Prospective Investigation into Cancer and Nutrition* [74] confirmed a positive relationship between milk intake in 2,109 European women with increased IGF-1 serum levels. An increase in serum IGF-1 concentration of more than 20 % has also been observed in prepubertal children not used to milk consumption after a 4-week intake of 710 mL of whole milk daily [75]. A recent study including 193 overweight adolescents aged 12–15 years drank either 1 L/day of skimmed milk, whey, casein or water for 12 weeks. All milk-based-drinks contained 35 g milk protein/L. IGF-1 significantly increased with skimmed milk and tended to increase with casein compared to the pre-test control group [76]. Casein in comparison to whey protein has been shown to differentially enhance hepatic IGF-1 synthesis [58] (Fig. 18.1). After the widespread implementation of household cooling technology in the 1950s per capita cheese consumption, the major dairy source of casein, increased in Germany by the factor 5 from 5 kg in the early 1950s to nearly 24 kg in 2013 [77]. Taken together, milk consumption has a profound IGF-1-enhancing effect on prepubertal, pubertal and adult humans, thus increases serum IGF-1 levels of young adult women in the period of reproduction.

Milk-Derived Amino Acids Activate mTORC1

Milk proteins in comparison to all other animal proteins provide highest amounts of essential BCAAs, especially highest amounts of leucine [64]. Milk protein (8.09 g glutamine/100 g) in comparison to beef protein (4.75 g glutamine/100 g) provides 70 % more glutamine than beef [78]. Glutamine is an important activator of mTORC1 signaling via (1) its function as a gatekeeper for the uptake of the mTORC1-activating amino acid leucine [28] and (2) via its availability

as a precursor of the glutaminolysis pathway that activates mTORC1 [42]. Furthermore, leucine is an allosteric activator of glutamate dehydrogenase (GDH), the key regulating enzyme of the glutaminolysis pathway [79]. On the other hand, GDH contributes to leucine sensing in the regulation of autophagy by activating mTORC1 [80]. Remarkably, the combination of glutamine and leucine, which maximizes the flux through GDH, is most effective in phosphorylation of S6K in β-cells [81]. Thus, leucine and glutamine, two predominant amino acids of cow's milk, interact at most critical steps of amino acid signaling resulting in the upregulation of mTORC1.

Milk-Derived Palmitate Activates mTORC1

Bovine milk contains about 3.5–5 % total lipid, existing as emulsified globules 2–4 μm in diameter and coated with a membrane derived from the secreting cell. In homogenized milk, the coat is mostly casein. About 98 % or more of the lipid is triacylglycerol, which is found in the globule [82]. The major fatty acid of the total fatty acid composition of bovine milk lipids is palmitate (C16:0) with 32.3 wt.% [82, 83]. Palmitate like BCAAs has recently been demonstrated to enhance the lysosomal translocation of mTORC1, a most critical step for the activation of mTORC1 [44].

In obesity and states of insulin resistance, in addition to increased serum levels of essential BCAAs [84–86], palmitate serum levels are significantly increased [87–89]. Thus, milk and dairy consumption in obese women enhances already elevated amino acid- and fatty acid-mediated signaling promoting the activation mTORC1.

Taken together, milk provides the ideal "cocktail of mammalian evolution" for the activation of mTORC1 by increasing insulin/IGF-1 signaling [90, 91], by providing essential BCAAs and abundant glutamine [64, 78], and by transferring palmitate, the fatty acid activator of mTORC1 [44, 82]. In fact, the activation of mTORC1 via measurement of increased phosphorylation of the mTORC1 target S6K1 in liver and white adipose tissue of mice fed on cow's milk has been demonstrated [92]. It has been reported that milk consumption increased body mass index and body weight in children [93, 94], adolescents [95], and adults [96]. Thus, the question arises whether milk protein intake in pregnancy may increase placental weight and upregulate mTORC1-mediated growth and nutrient transfer of the syncytiotrophoblast to the fetus. Furthermore, it will discussed whether the milk-induced increase of placenta mass increases the amount and signaling of placental hormones such as human placental growth hormone (PGH) and human placental lactogen (HPL).

Milk-Derived Glucose Activates mTORC1

Lactose is a disaccharide sugar derived from galactose and glucose and makes up around 5 % of milk by weight. Lactose is hydrolyzed by the enzyme lactase encoded by the *LCT* gene, which is physiologically expressed along the brush border membrane of differentiated enterocytes lining the villi of the small intestine during the period of milk feeding [97]. Since lactase's only function is the digestion of lactose in milk, in most mammalian species the activity of lactase is dramatically reduced after weaning [98]. Lactase persistence has resulted from mutations of the *LCT* gene that allowed persistent milk consumption in European and African populations [99, 100]. However, the "original" genetic design of mammalian biology is exclusive and species-specific milk consumption restricted to the postnatal growth phase until weaning. Lactase persistent pregnant women who may consume 1 L cow's milk are thus able to metabolize approximately 25 g of glucose, a substantial amount that may further activate milk-mediated mTORC1 signaling.

Milk Consumption in Pregnancy Increases Placental, Fetal and Birth Weight

As milk promotes mTORC1 signaling [2] and has been demonstrated to increase linear growth in children [101] as well as BMI in

children, adolescents and adults [93–96], it is most conceivable that increased milk consumption may influence placental growth as well. Data from 50,117 mother-infant pairs of the *Danish National Birth Cohort* collected from 1996 to 2002 showed a placental weight increase across the whole range of milk intake [102]. A linear increment of placental weight from 13.3 g (0–1 glass of milk/day) to 26.4 g (>6 glasses of milk/day) ($p < 0.001$) has been reported.

An increase in placental weight may not only increase the amount and capacity of nutrient transporters for nutrient transfer to the fetus but may also increase the amount of placenta-derived growth hormones that may decrease maternal insulin sensitivity thereby raising maternal blood glucose levels promoting fetal growth and birth weight. The *Generation R Study*, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam investigated 3,405 mothers during pregnancy. Maternal milk consumption of >3 glasses (450 mL of milk) per day was associated with greater fetal weight gain in the third trimester of pregnancy, which led to an 88 g higher birth weight than that with milk consumption of 0–1 glass per day [103]. Intriguingly, maternal protein intake, but not fat or carbohydrate intake, from dairy products was associated with higher birth weight [103]. This association was limited to milk, whereas protein intake from nondairy food or cheese was not associated with birth weight. Compared with the lowest reference category of milk consumption (0–1 glasses/day), maternal milk intakes of >1–2 glasses/day, 2–3 glasses/day, and >3 glasses/day was associated with increased fetal weight gain. Fetal weight gain has been estimated by morphometric ultrasound examinations during pregnancy according to the procedure of Hadlock et al. [104]. Milk-mediated differences in fetal weight gain appeared from 20th week onward, but became most evident in the last part of the third trimester [103]. There are other international studies, which report an increase of birth weight in relation to milk consumption during pregnancy (Table 18.1). A retrospective cohort in Sweden reported a birth weight increase of 75 g and 134 g in the offspring of mothers consuming >200 mL and 1 L milk daily, respectively [105]. A prospective study in India reported that the frequency of milk consumption at 18th week of gestation was positively associated with placental weight, birth weight, birth length, and head circumference [106]. According to a prospective study in Canada, maternal daily consumption of an additional 1 cup (237 mL) of milk was associated with a 41 g increase in offspring birth weight [107]. A prospective Australian study in 557 mothers reported that protein intake from dairy products was associated with a higher birth weight of the offspring [108]. In a randomized controlled trial of 72 adolescent pregnant mothers, 25 mothers were counseled to consume >4 servings of dairy products a day, which resulted in a 240 g higher birth weight in this group compared to the control group [109]. According to a recent systematic literature review, the majority of studies reported positive associations between milk and/or dairy consumption and birth weight-related outcomes [110].

Taken together, accumulating evidence underlines that milk consumption during pregnancy increases placental weight, fetal growth and birth weight as well as birth size (Table 18.1). Noteworthy, significant and dose-dependent increase in fetal growth and birth weight is associated with milk protein intake during pregnancy, whereas no effect was observed with cheese protein intake [102, 103]. Future studies should elucidate the differential contribution of the whey protein and the casein fraction of milk on the growth promoting effects of the placenta and fetal growth. In comparison to caseins, which clot in the stomach, whey proteins are fast proteins that are easily hydrolyzed in the intestine and deliver immediate “bursts” of BCAAs [57–59]. Whey-derived insulinotropic BCAA pulses apparently induce postprandial hyperinsulinemia [60, 64–69]. Notably, whey-induced BCAA and insulin pulses may evoke exaggerated effects on syncytiotrophoblast mTORC1 signaling.

Table 18.1 Impact of milk consumption during pregnancy on maternal-fetal weight parameters

Effect of milk intake	Outcome	Studies [references]
Pre-pregnancy body weight	Increase	Randomized intervention study, Denmark [95]
Gestational weight gain	Increase	Observational study, Iceland [192]
Placenta weight	Increase	Danish National Birth Cohort, Denmark [102]
		Pune Maternal Nutrition Study, India [106]
Fetal weight	Increase	Generation R Study, Netherlands [103]
Birth weight	Increase	Generation R Study, Netherlands [103]
		Observational study, Sweden [105]
		Pune Maternal Nutrition Study, India [106]
		Prospective observational study, Australia [108]
		Randomized controlled trial, USA [109]
		Systematic literature review, Norway [110]

Milk Consumption Placental Weight Gain and Growth Hormone Signaling

Milk-induced weight gain of the placenta may modify the synthesis and amount of placental growth hormones. Changes in fetal growth associated with differential gene expression of PGH and HPL (also called chorionic somatomammotropin, CSH) may be modulated by maternal nutrinonal status [111]. CSH1, the predominant transcript of HPL is increased in placentas of large for gestational age (LGA) pregnancies [111]. Notably, maternal blood levels of HPL are correlated with placenta weight [112–114] and fetal weight [114–117]. Moreover, a link between fetal growth velocities in the second half of the pregnancy and changes in HPL in maternal serum has been demonstrated [118]. In LGA newborns the expression of *CSH1-1*, *CSH2-1*, and *CSHL1-4* mRNA transcripts in placenta was significantly increased compared with appropriate for gestational age (AGA) newborns [119]. Women with LGA newborns had an increased BMI before pregnancy (25 kg/m^2), an increased gestational weight gain (19 kg), and placental weight (777.6 g) compared to AGA newborns associated with a normal maternal BMI before pregnancy of 22.4 kg/m^2, a gestational weight gain of 15.8 kg, and a placental weight of 650 g, respectively [119].

Recent evidence indicates that maternal serum PGH is positively associated with fetal growth in the first half of pregnancy [120]. It is not known whether PGH correlates with placental weight. However, PGH acts to augment the supply of fatty acids and glucose to the placenta by the induction of insulin resistance [121–123]. Several studies suggest an important role for both PGH and HPL in the control of fetal growth [124–126].

Milk- and Placental Growth Hormone-Induced Insulin Resistance

The somatogenic and lactogenic hormones of the placenta and maternal pituitary gland integrate the metabolic adaptations of pregnancy with the demands of fetal and neonatal development. Dysregulation of PGH and/or HPL in pathologic conditions of pregnancy may adversely impact fetal growth and postnatal metabolic function [127]. In addition to promoting growth of maternal tissues, PGH induces maternal insulin resistance and thereby facilitates the mobilization of maternal nutrients for fetal growth. HPL and prolactin increase maternal food intake by induction of central leptin resistance and promote maternal β-cell expansion and insulin production [127]. Maternal insulin resistance increases serum glucose levels and thus the glucose supply to the placenta.

Milk is the animal protein source providing the highest amounts of essential BCAAs [64],

which have recently been linked with the development of insulin resistance [84–87]. It has been shown in children, who in comparison to adults exhibit increased pituitary GH levels, that milk consumption increases serum insulin, GH and IGF-1 levels and induces insulin resistance [70, 75]. GH and PGH bind and activate the maternal GH receptor (GHR), whereas HPL activates the maternal prolactin receptor (PRLR) [128]. Both receptor pathways via activation of signal transducer and activator of transcription 5 (STAT5) induce the expression of suppressor of cytokine signaling proteins (SOCS) [128]. GH induces the expression of SOCS2 and SOCS3, whereas HPL induces SOCS1 and SOCS2 [120]. SOCS1, SOCS3, SOCS6 and SOCS7 are negative regulators of insulin signaling [129–131]. SOCS proteins inhibit insulin receptor signaling by binding to the insulin receptor (IR), thereby blocking access of signaling intermediates and inhibiting insulin receptor tyrosine kinase activity, leading to a reduction of IR-directed phosphorylation of insulin receptor substrate-1 (IRS-1) and its downstream events, and by targeting IRS-1 and IRS-2 for proteasomal degradation [129, 130]. PGH and HPL signaling via SOCS expression thus induce SOCS-mediated insulin resistance [129–132]. In pregnancy, PGH- and HPL-mediated maternal insulin resistance is necessary for adequate glucose supply to the fetus. However, uncontrolled SOCS-mediated insulin resistance will result in gestational diabetes mellitus (GDM).

Moreover, the whey component of milk increases GIP signaling [64–66]. GIP receptors are not only expressed by pancreatic β-cells and GIP may not only signal via the entero-insular axis stimulating insulin secretion but also enhances GH secretion of the somatotroph cells of the pituitary, which express the GIP-receptor (GIPR) [133]. GIPR activation elevates cAMP, which drives GH-promoter activity [133]. As GHR signaling controls the expression SOCS2 and SOCS3, consumption of whey-containing milk products may overstimulate SOCS-induced maternal insulin resistance and may contribute to increased glucose flux to the fetus overstimulating fetal growth. Thus, extensive milk consumption during pregnancy may increase the risk for GDM.

In milk consuming pregnant women, placental growth hormone signaling overlaps with milk signaling, which apparently via mTORC1-activation and S6K1-mediated phosphorylation of IRS-1 and SOCS-mediated degradation of IRS-1 reduce insulin signaling of the pregnant mother. BCAAs by increasing mTORC1-S6K1 signaling act as positive signals for maintenance of protein stores, while inhibiting other actions of insulin at multiple levels [134]. In amino acid-infused humans, over-activation of mTORC1-S6K1 pathway increased inhibitory IRS-1 phosphorylation at Ser312, Ser636/639 and Ser1101 resulting in insulin resistance of skeletal muscle [135–137]. Thus, there is substantial evidence that inappropriate activation of mTORC1-S6K1 signaling by amino acids induces insulin resistance, the fundamental metabolic deviation leading to type 2 diabetes mellitus and GDM [135–138]. Whey proteins in contrast to meat proteins provide fast hydrolysable BCAAs mimicking a BCAA infusion that promotes insulin secretion and insulin resistance, major intrinsic mechanisms of milk signaling [2].

Both, milk-mediated mTORC1-induced insulin resistance and HPL-mediated insulin resistance may synergistically overstimulate the physiological insulin resistance of pregnancy. Furthermore, milk signaling and PGH signaling increase maternal hepatic IGF-1 synthesis [73–75, 126]. Chellakooty et al. [126] observed a positive association between the change in PGH and the change in IGF-1 levels throughout gestation. The change in IGF-1 throughout gestation was significantly positively associated with placental weight at birth [126]. Thus, the IGF-1-increasing effect of milk consumption may result in an additional increase of placental weight, which in fact has been confirmed epidemiologically [102].

In mammals the growth and insulin resistance promoting system milk is intended to induce and maintain postnatal growth and is never combined with the insulin resistance promoting placental growth hormone system of pregnancy except for milk-consuming pregnant women.

Milk Consumption and FGF21-Mediated Over-Expression of GLUT1

Cow's milk consumption during pregnancy increases placental weight [102]. Notably, placental weight is associated with increased maternal serum levels of HPL [112–114]. Lactogens, especially HPL, have been demonstrated to activate the Janus-activated-kinase-2 (JAK2)/signal transducer and activator of transcription-5 (STAT5) signaling pathway [139–141]. Increased circulating serum levels of the recently discovered fibroblast growth factor 21 (FGF21) have been related with insulin resistance, type 2 diabetes, obesity and metabolic syndrome [142, 143]. In comparison to control subjects, plasma FGF21 levels were significantly higher in women with GDM [144]. Dekker Nitert et al. [145] demonstrated that FGF21 is expressed in the placenta. FGF21 mRNA expression is increased in women with GDM [145]. Notably, the FGF21 promoter contains three putative STAT5-binding sites [146]. It has been shown that GH increased STAT5 binding to the FGF21 promoter and enhanced FGF21 expression in the liver [146]. Increased FGF21 production has been observed in late pregnancy in the mouse [147]. In mice, GH-induced hepatic FGF21 production is stimulated by free fatty acid (FFA)-mediated activation of PPARα [148]. In neonatal mice, milk intake via FFA-mediated stimulation of PPARα enhanced hepatic FGF21 expression [149]. Furthermore, recent evidence indicates that hepatic mTORC1 signaling regulates the expression of FGF21. Ectopic activation of hepatic mTORC1 in liver-specific *Tsc1* knockout mice resulted in peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α)-dependent expression of FGF21 [150]. Intriguingly, overexpression of FGF21 in 3T3-L1 adipocytes upregulated glucose uptake rates and increased the mRNA expression of glucose transporter 1 (GLUT1) [151].

GLUT1 is believed to be the primary glucose transporter isoform in the human placenta and increases its expression over gestation [152]. GLUT1 has been localized to both the maternal facing microvillous plasma membrane (MVM) with threefold higher expression as compared to the basal plasma membrane (BM) [153]. In maternal diabetes, the expression of GLUT1 in the BM has been reported to increase [154, 155]. Notably, increased syncytiotrophoblast BM expression of GLUT1 has been associated with increased birth weight of large babies of non-diabetic mothers [156].

Thus, milk-activated mTORC1 signaling via mTORC1-mediated overexpression of FGF21 and enhanced HPL/STAT5-mediated expression of placental FGF21 may overstimulate the expression of basal membrane GLUT1, which increases glucose flux to the fetal capillaries, thus promotes fetal weight gain. Taken together, there is good reason to assume that milk consumption during pregnancy enhances PGH/HPL-STAT5 signaling, milk-derived FFA-PPARα-signaling, and milk-driven mTORC1 signaling that may overstimulate placental FGF21-mediated GLUT1-expression enhancing glucose transport to the fetus.

Potential Role of Milk miR-21 on Fetal Growth

Exosomal microRNA-21 (miR-21) is a consistent component of cow's milk [157]. miR-21 appears to play a key role in mTORC1 signaling. Critical targets of miR-21 are mRNAs of important tumor suppressor proteins involved in upstream and downstream suppression of mTORC1 signaling, i.e., PTEN [158–161], Sprouty1 and Sprouty2 [162–164], PDCD4 [165–167]. Furthermore, miR-21 has been shown to induce the cell cycle promoter cyclin D1 in an mTORC1-dependent manner [168]. Supposed that milk-derived miR-21 reaches distant cells of the milk recipient, PTEN suppression could increase insulin/IGF-1/PI3K/AKT signaling, which further augments mTORC1 activation. MiR-21-induced inhibition of Sprouty1 and 2 would amplify RAS-RAF-MEK-ERK signaling, which additionally suppresses TSC2 and thus raises mTORC1 activity. Furthermore, miR-21 could stimulate the initiation of translation by

repression of PDCD4, which is a suppressor of translation initiation that inhibits the RNA helicase eIF4A [169]. Both, 4E-BP-1 and PDCD4 are crucial regulatory inhibitors of translation initiation and thus of protein synthesis. Activation of the mTORC1 pathway and its substrate kinase S6K1 results in subsequent phosphorylation of 4E-BP-1 and PDCD4 that promote eIF4E-eIF4G complex assembly and stimulate mRNA translation [168]. MiR-21-mediated suppression of PDCD4 expression may further amply translation initiation, a reasonable regulatory step of milk signaling to promote postnatal growth. Intriguingly, Jiang et al. [170] recently reported on aberrant upregulation of miR-21 in placental tissue of macrosomia. Among others the target genes of miR-21 were involved in JAK/STAT- and the mTORC1 signaling pathways [170]. Future research should clarify whether milk-derived miR-21 may contribute to the development of mTORC1-driven fetal macrosomia.

The Role of mTORC1 in Placental Nutrient Transfer

The nutrient-sensitive kinase mTORC1 regulates cell metabolism and growth in response to altered nutrient levels and growth factor signaling [4–11]. During gestation the placenta integrates and regulates nutrient transfer to the fetus required for fetal growth [171, 172]. The syncytiotrophoblast represents the transporting epithelium and the primary endocrine cell of the human placenta, which plays the most critical role in determining fetal growth (Fig. 18.1). Trophoblast cell metabolism and associated signaling influence fetal nutrient availability. Trophoblast nutrient sensors may have a unique role in regulating fetal growth. Recent evidence underlines a pivotal role of mTORC1 in placental nutrient sensing [173]. mTOR is highly expressed in the syncytiotrophoblast of the human placenta [174]. It has been shown in cultured primary human trophoblast cells that mTORC1 is regulated by glucose, amino acid- and growth factor signaling [175]. Placental insulin/IGF-1 signaling and fetal levels of oxygen, glucose and amino acids are altered in pregnancy complications such as intrauterine growth restriction, and all these factors are well-established upstream regulators of mTORC1 [4–11]. Furthermore, mTORC1 is a positive regulator of placental system A and system L amino acid transporters, suggesting that trophoblast mTORC1 modulates amino acid transfer across the placenta [173]. Syncytiotrophoblast mTORC1 activation increases cell surface density of amino acid transporters in the trophoblast and thus links maternal nutrient availability and growth factor signaling to fetal growth by modulating mTORC1-mediated flux of amino acids across the placenta finally stimulating amino acid-mediated mTORC1 activation of fetal cells for growth [173]. In this regard, cow's milk consumption during pregnancy may enhance the magnitude of trophoblast mTORC1 signaling. Milk consumption increases maternal blood levels of insulin and IGF-1 and increases the amount of essential BCAAs, glutamine and palmitate, all important nutrient and growth factor signals activating mTORC1 [44, 53–57, 60, 66, 73–75].

Placental mTORC1 signaling is compromised in pregnancy complications associated with altered fetal growth. It has ben shown that mTORC1 in the human placenta is downregulated in restricted fetal growth [174]. Kavitha et al. [13] recently demonstrated in pregnant baboons that maternal nutrient restriction downregulated placental mTOR, insulin/IGF-1 signaling and nutrient transporters.

In contrast, activation of placental mTORC1 signaling has been observed in association with maternal obesity [176]. In female Albino Wistar rats maternal overweight induced by a diet with high content of saturated fat activates placental mTOR and eIF2α signaling and increases fetal growth [177]. Obesity is associated with elevated circulating levels free palmitate [178] and hyperleucinemia, hyperinsulinemia and insulin resistance [71, 84–86]. Thus, obesity exhibits a metabolic environment with enhanced insulin, BCAA, glucose and palmitate signals that overstimulate trophoblast mTORC1 activity. In obese women giving birth to LGA newborns the activity of placental insulin/IGF-1 and mTORC1 signaling was positively correlated with birth weight [176].

Milk consumption during pregnancy thus exaggerates obesity-mediated mTORC1-activating conditions of the trophoblast by increasing insulin/IGF-1 signaling and providing abundant amounts of BCAAs and glutamine, all converging in the activation of placental mTORC1. This mechanistic view clearly supports the adverse impact of milk consumption during pregnancy in the promotion of placental and fetal overgrowth as confirmed by epidemiological data [101, 102].

Milk Consumption Increases Maternal Weight

Rapid weight gain in early prenatal life, increased birth weight and accelerated weight gain during early postnatal life exert adverse effects of nutritional and hormone-dependent programming [179–182] and are associated with increased risk of obesity, diabetes [183–185], asthma [186], hypertension, cardiovascular diseases [181, 187], and cancer [188, 189]. Epidemiological studies in human and animal experiments showed that nutrition during fetal and neonatal life promote diseases of civilization in adulthood. The overall effects of the maternal environment on the placenta are the product of its exposures throughout gestation, the "placental exposome" [190]. Milk-derived BCAAs, insulin, IGF-1 and mTORC1 signaling most likely result in significant adverse alterations of the placental exposome.

Maternal overweight and obesity during pregnancy are associated with higher birth weights and more body fat of the offspring [191]. Arnberg et al. [95] investigated 203 overweight adolescents with a BMI of 25.4 ± 2.3 kg/m^2 (mean ± SD), who received an additional daily amount of 35 g milk protein either as 1 L/d of skim milk, whey, or casein, or water as a control for 12 weeks. BMI-for-age Z-score was greater at 12 weeks in the skim milk, whey, and casein groups compared with baseline and with the water and pretest control groups. Thus, milk consumption increased body weight of adolescents. Intriguingly, pregnant women gaining excessive weight gain in comparison to women gaining optimal weight gain reported a twofold intake of dairy products of about 200 g/day [192]. Of all dairy products, dairy protein and dairy fat, the strongest predictor of increased maternal weight gain during the last trimester of pregnancy was milk [192]. Thus, milk consumption during pregnancy contributes significantly to increased maternal weight. Babies of obese women are often large at birth [193–197]. Jansson et al. [176] recently demonstrated that the activity of placental insulin/IGF-1 and mTORC1 signaling was positively correlated to birth weight. Milk signaling during pregnancy by high cow's milk intake appears to exaggerate imbalanced metabolic and hormonal signaling of obesity and diabetes during pregnancy.

Conclusions and Outlook

Milk, is not just food but represents a specific postnatal programming system for postnatal growth, a powerful nutritional cocktail of mammalian evolution that drives the adequate growth axis of the newborn infant [2]. Cow's milk supports the growth of *Bos taurus*, a species that doubles birth weight four times faster than human infants [198]. The persistent intake of bovine milk, a secretory product promoting species-specific postnatal growth, by humans during pregnancy is an absolute biological exception not observed in any other mammalian species. No adult pregnant mammal will be exposed to the signaling system of another species' milk. Cow's milk consumption during pregnancy promotes weight gain in the pre-pregnancy period [95], during pregnancy, especially in the third trimenon [192], increases placental weight [102, 106], increases fetal growth especially in the third trimenon [103, 192] and consecutively increases birth weight of the offspring [105, 106, 108–110] (Table 18.1). There is accumulating evidence that milk consumption during childhood and adolescence increases BMI and body weight [92–96], thus may result in unfavorable pre-pregnancy conditions that adversely affect weight gain during pregnancy [195]. Milk consumption during pregnancy accelerates both placental and fetal growth [102, 190]. There is good reason to

assume that cow's milk-driven mTORC1 signaling during pregnancy exaggerates placental mTORC1 signaling. The milk-induced increase of placental weight may be associated with increased serum levels of HPL, which via induction of SOCS1/SOCS2 signaling promote maternal insulin resistance. Milk-driven maternal mTORC1 signaling via activation of S6K1 may further impair maternal insulin-IRS-1 signaling thereby enhancing the materno-fetal glucose gradient, which leads to overstimulating fetal growth. Moreover, milk-mediated overstimulation of mTORC1 signaling may directly upregulate syncytiotrophoblast amino acid transporter expression [173–176], thus increasing the transfer of amino acids to the fetus (Fig. 18.1). High glucose and amino acids (BCAAs) levels finally promote mTORC1 signaling of fetal cells resulting in fetal overgrowth and macrosomia.

Milk, the mTORC1 promoting "starter kit" of postnatal life represents an underestimated risk factor with significant impact on the placental exposome. It is thus of critical concern that gynecologists frequently recommend milk and dairy protein consumption during pregnancy as a rich source of calcium "to improve" fetal bone mineralization. Milk unfortunately not only delivers abundant calcium but most critically transfers a signaling system that increases the magnitude of mTORC1 signaling of the milk recipient and her fetus. Overstimulated mTORC1-mediated metabolic programming during the perinatal period has negative effects on lifelong metabolic and immunological programming [179–184, 197–200], which are intimately involved in the pathogenesis of mTORC1-driven age-related diseases of civilization [37, 201, 202]. Modern concepts of mTOR biology lead to the conclusion that only appropriate maternal, placental and fetal mTORC1 signaling guarantees healthy metabolic programming. Accelerated mTORC1 signaling as a consecutive reaction pattern of intrauterine growth restriction as well as over-stimulated perinatal mTORC1 signaling by overnutrition, especially milk consumption may adversely affect health and life span.

Milk is nature's design for postnatal mammalian growth but not for fetal growth. In terms of anthropological biology, the introduction of milk consumption since the Neolithic revolution [203], promoted by refrigerator mass production since the 1920s in industrialized societies, is a fundamental change of human behavior bearing the risk of longterm adverse effects on human health [1]. Cow's milk consumption during pregnancy and cow's milk protein-based artificial infant formula feeding deviate perinatal axes of mTORC1 signaling and accelerate perinatal growth. In 1934, McCay and Crowell [204] from Cornell University provided translational evidence that slow growth favors longevity. Milk consumption during pregnancy disturbs a most critical period of perinatal growth, which may result in irreversible changes in the developmental trajectory [205]. Contemporary urban women of the "latte macchiato/cappuccino generation" are already exposed to increased intake of milk. These pregnant women are further encouraged by their obstetricians and gynecologists to increase their milk and dairy consumption with the intention to provide sufficient amounts of calcium [206]. However, it is of most critical concern that these recommendations stress milk signaling during pregnancy, a most sensitive period of lifelong mTORC1-driven metabolic programming. Milk, a signaling system for postnatal growth, should not interfere with prenatal growth and should not be recommended for pregnant women. Milk consumption during pregnancy appears to be a yet underestimated health hazard for the human fetus.

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Genetic Disease Specific Human Embryonic Stem Cell Lines

19

Anver Kuliev, O. Verlinsky, and S. Rechitsky

Introduction

Preimplantation Genetic Diagnosis (PGD) provides unlimited source for obtaining human embryonic stem cell (hESC) lines. As PGD involves the pre-selection of the genetic disease – free embryos for transfer back to uterus the affected embryos were either discarded, or used for confirmation of diagnosis. These affected embryos otherwise provided the valuable source for the derivation of the genetic disease derivation of hESC lines with genetic and chromosomal disorders, which will be described in this paper. There were also attempts to obtain hESC lines from a single cell, removed from the 8-cell stage embryo at the time of embryo biopsy for PGD purposes, so these lines could be used for transplantation purposes even before the baby is born and can be a potential donor of HLA matched stem cells for the affected sibling [1, 2]. Despite feasibility of this approach, it does not seem practical, as removing additional material from the embryos at this stage may compromise the viability of the embryo.

Feasibility of establishing hESC lines from the inner cell mass of the blastocyst was first demonstrated in 1998 [3], and presently hESC lines for research purposes are provided by the NIH repository of hESC lines. This registry initially contained 78 lines, of which only 11 have met NIH scientific criteria, including the presence of L-alkaline phosphatase (TRA-2-39), Oct-4, high molecular weight glycoproteins (antibodies TRA-1-60, TRA-1-81), stage specific embryonic antigens (SSEA-3, SSEA-4), euploid karyotype and teratoma formation in SCID mice [4]. The list of hESC lines in the NIH registry is currently being extended, following Executive Order 13505, entitled "Removing Barriers to Responsible Research Involving Human Stem Cells." The Order was issued by President Obama on March 9, 2009, lifting the ban regarding the use of Federal funds for hESC research. Although the NIH registry has been significantly expanded, it contains only a few hESC lines with different genetic abnormalities I (see below).

The main source for obtaining hESC lines in our collection was the blastocyst stage embryo inner cell mass (ICM), isolated by immunosurgery and placed on a feeder layer, although other sources, such as morula was also attempted but appeared impractical [5–8]. The established hESC lines were tested for alkaline phosphatase, stage specific antigens SSEA-3 and SSEA-4, high molecular weight glycoproteins or tumour rejection antigens, TRA-1-60 and Oct-4. They were maintained in vitro from 10 to 15 passages before freezing in sufficient amounts, with no

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observation of differences in the efficiency of obtaining hESC lines depending on the source.

Our collection of the hESC lines with genetic and chromosomal disorders, described in this paper, contains 87 lines, including 14 with chromosomal abnormalities and 73 with monogenic conditions, which are frozen and are available at different passages.

Chromosomal Disease Specific Human Embryonic Stem Cell Lines

The list of the chromosomal disease specific hESC lines is presented in Table 19.1. These are however only those few lines that were established, while the majority of attempts failed due to a poor outcome of hESC lines from the embryos with autosomal aneuploidies. Also, because of selective disadvantage of abnormal cells in culture, some of the hESC lines attempted to derive from chromosomally abnormal embryos ended up having a normal karyotype, phenomenon observed also reported previously [9–11]. On the other hand, it was reported that there is a risk of de novo chromosomal abnormalities in the process of the propagation and maintenance of hESC lines [12]. One of such incidental chromosomal abnormalities was detected in the hESC line, distributed from NIH registry to more than 100 research laboratories around the world. This hESC line was obtained from the Wisconsin-based stem cell registry WiCell, and have had a normal female karyotype, which was stable though the establishment and maintenance for several months, with the ability to differentiate with the formation of neural and beating cardiac muscle cells. Karyotyping changes, involving the gain of the chromosome 17q, were observed in three independent hESC lines on the five independent occasions, together with the occasional gain of chromosome 12, which was suggested to be attributable to a selective advantage of these cells to the propagation of undifferentiated cells. This phenomenon created a concern over the use of hESC lines for hESC -based therapies, because cytogenetic changes may be only the part of genetic abnormalities acquired in the process of the establishment, maintenance and differentiation of hESC lines.

Table 19.1 Chromosomal disease-specific hESC lines (total 14 lines)

	Stem cell karyotype
1	69,XXY
2	47,XXY
3	46,XX, der(4) t(4;13)
4	47,XX,+13
5	47,XX,+14
6	47,XXY
7	47,XXX
8	47,XY + 12
9	47,XY + der(21)t(2,21)
10	46,X + MAR
11	46,XX (T14;17)
12	47,XX + 21
13	46,XX iso (17q)
14	47,XX + 12

In a special multicenter study of 127 hESC lines from 39 laboratories worldwide for genetic changes occurring during culture, it was demonstrated that although these lines remained cytogenetically normal, a progressive tendency to acquire karyotypic changes on prolonged culture were also observed, commonly affecting chromosomes 1, 12, 17 and 20 [13]. Analysis for single nucleotide polymorphisms (SNP) showed that there were also genomic structural variants, which appeared sporadically but no common variants related to culture conditions were observed on chromosomes 1, 12 and 17. However, overlapping structural variants were acquired on chromosome 20p11.21 during culture of multiple cell lines, suggesting the anti-apoptotic gene, BCL2L1, as the most likely driver of culture adaptation in this chromosomal region. This makes initial genetic testing of the embryos used as a source of ESC lines one of the basic requirements.

A few hESC lines with chromosomal disorders were reported previously, including lines with trisomy 13, triploidy and mosaicism ([14]; NIH Registry 2011). As presented in Table 19.1, our collection of hESC lines contains 14 hESC lines obtained from the embryos with chromosomal disorders, including 4 lines with translocations, 1 trisomy 14, 1 triploidy, 1 trisomy 13, 2

trisomy 12, 1 trisomy 21, and 4 with aneuploidy of sex chromosomes (1 with 45, X+mar, 1 with 47, XXX and 2 with 47, XXY, one of which was derived from the same embryo that was the source of the hESC line with EmeryDreifuss (carrier) type muscular dystrophy (see below).

However, as mentioned, this is only a small proportion of those embryos from which hESC lines were obtained, as the majority of the attempts failed. In fact, not all embryos are able even to reach the blastocyst stage, to allow attempting the obtain hESC lines, and this also may depend on the origin of aneuploidy, that affects the derivation success rates. We have previously demonstrated that the plating efficiency differed significantly depending on the origin of aneuploidy, with very low plating efficiency, when the embryos with prezygotic aneuploidy were the source, such as those originating from the zygotes with MI or MII errors [15]. Karyotypically normal hESC lines may be also produced from the embryos with chromosomal abnormalities [9–11], but undetected mosaicism could not be excluded in these cases, as the diagnosis was obtained at the cleavage stage that is characterized by an extremely high mosaicism rate, so selective advantage of normal cell in such cases may have taken place.

Table 19.2 Autosimal recessive disease-specific hESC lines: total 24 lines

Hemoglobin – alpha locus; HBA, affected (– – / – –)
Hemoglobin – beta locus; HBB, affected (cd39/IVS1-110)
Hemoglobin – beta locus; HBB, affected (cd8 + G/619del)
Hemoglobin – beta locus; HBB, affected (HbS/HbS – sickle cell anemia)
Hemoglobin – beta locus; HBB, affected (IVS1-5/Cd8 + G)
Hemoglobin – beta locus; HBB, affected (IVSI-6/IVSI-6)
Hemoglobin – beta locus; HBB, affected (Unknown/IVSII-1)
Hemoglobin – beta locus; HBB, affected (Unknown/IVSII-1)
Hemoglobin – beta locus; HBB, carrier (N/IVS 1-1)
Hemoglobin – beta locus; HBB, carrier (N/ IVS1-110)
Cystic fibrosis; affected (ΔF508/1717–1 G > A)
Cystic fibrosis; affected (ΔF508/1717–1 G > A)
Cystic fibrosis; affected (ΔF508/ΔF508)
Cystic fibrosis; affected (ΔF508/ΔF508)
Cystic fibrosis; affected (ΔF508/ΔF508)
Cystic fibrosis; affected (ΔF508/ΔF508)
Cystic fibrosis; affected (N1303K/ΔF508)
Cystic fibrosis; affected (W1282X/R117C)
Fanconi anemia, complementation group A; FANCA, carrier of 14 bp deletion, spinal muscular atrophy, type I; SMA1, affected, exon 7 deletion ($n=2$)
Sandhoff disease, affected (n = 3)

Genetic Disease Specific Human Embryonic Stem Cell Lines

Tables 19.2, 19.3, and 19.4 present our collection of the monogenetic disease specific hESC lines, representing the world's largest repository with 73 hESC lines with genetic disorders [16–19]. These lines provide an unlimited source for understanding the mechanisms of the phenotypic realization of genetic defects and for the development of the approaches for their possible treatment. A few hESC lines with genetic disorders are available in NIH registry, and were reported also by other laboratories, including cystic fibrosis, Charcot-Marie-Tooth disease, Duchenne Muscular Distrophy, congenital nephrotic syndrome, spinal muscular atrophy, and Marfan syndrome ([20]; NIH Stem Cell Registry).

The success rate of derivation of the genetic disease specific hESC lines was 20–25 %, not different from the success rate observed for hESC lines obtained from normal embryos. As seen from Table 19.2, our collection of hESC lines with genetic disorders contains 24 hESC lines derived from the embryos with autosomal recessive disorders, including 10 with beta-globin mutations (including thalassaemia and with sickle cell disease), 1 Fanconi anaemia, complementation group A, 8 cystic fibrosis, 2 spinal muscular atrophy, 3 Sandhoff disease, and 1 spinal muscular atrophy.

Fourteen hESC lines were obtained from the embryos with X-linked disorders (Table 19.3), including 1 adrenoreukodystrophy, 2 fragile site mental retardation (one affected male and one carrier female), 2 ocular albinism, 1 Becker, 4

Table 19.3 X-linked disease-specific hESC lines: total 14 lines

Albinism, ocular, type I; OA1, (c.251del C), affected male (1 line)
Albinism, ocular, type I; OA1, (N/c.251del C), carrier (1 line)
Adrenoleukodystrophy; ALD, (1801 del AG) affected male (1 line)
Muscular dystrophy, Becker type; BMD, affected male (1 line)
Muscular dystrophy, Duchenne type; DMD, affected (2 lines)
Muscular dystrophy, Duchenne type; DMD, carrier (2 lines)
Emery-Dreifuss muscular dystrophy, X-linked; EDMD, affected male (3 lines)
Emery-Dreifuss muscular dystrophy, X-linked; EDMD, carrier (1 line)
Fragile site mental retardation 1 affected male, expansion (1 line)
Fragile site mental retardation 1, carrier female (1 line)

Table 19.4 Dominant disease specific hESC lines: total 35 lines

Breast cancer, familial (brca2); affected (n/ivs7 gt del) (1 line)
Breast cancer, familial (brca2); affected (n/ivs7 gt del) and multiple endocrine
Neoplasia, type I; men1 affected (n/3036 4 bp del) (1 line)
Huntington disease; HD, affected, expansion (7 lines)
Marfan syndrome; MFS, affected, g7712a/n (1 line)
Dystrophia myotonica 1, affected, expansion (2 lines)
Neurofibromatosis, type I; NF, affected, (7 lines)
Torsion dystonia 1, autosomal dominant; DYT1, affected, exon 7 gag deletion (3 lines)
Treacher Collins-Franceschetti syndrome; TCOF, affected (nt. 4374 ins. a/n) (3 lines)
Tuberous sclerosis type 1, affected (2 lines)
Popliteal pterygium syndrome; PPS, affected (1 line)
Facioscapulohumeral muscular dystrophy 1a; FSHMD1A, affected (7 lines)

Duchenne (two affected and two carriers), and 4 Emery-Dreifuss (one affected and one carrier) type muscular dystrophy.

Thirty-five hESC lines were derived from the embryos with autosomal dominant conditions (Table 19.4), including 7 with neurofibromatosis type 1, 1 Marfan syndrome, 3 torsion dystonia, 2 tuberoses sclerosis, 1 pterigium syndrome, 7 fascioscapular muscular dystrophy 1A, 2 muscular dystrophy, 3 Treacher Collins-Franceschetti syndrome, 1 familial breast cancer BRCA-2, 1 familial breast cancer BRCA-2 together with type I multiple endocrine neoplasia and 7 Huntington's disease.

In addition, 12 hESC lines with polymorphism in the chemokine receptor 5 (CCR5) CMKBR5 gene, which in homozygous status confers resistance to HIV [21, 22], was detected by screening of 137 genetically normal hESC lines from our collection. One of them was established from the blastocyst deriving from partenogenetic embryo (46, XX), with two copies of all maternally derived genes, as demonstrated by polymorphic markers for X-chromosome, and chromosomes 3, 6, 11, 13, 18 and 21 [23]. Although this polymorphism was linked to HIV resistance long time ago, providing an immense promise of being able to treat HIV positive patients [21, 22], finding acceptable donor matches homozygous for the CCR5del32 presented a real challenge. So with the establishment of larger repositories of hESC lines, a search for finding HLA match for HIV patients may also be performed. At this time, the hESC lines with this polymorphism, and especially the one that has two copies of CCR5del32 allele, may provide an unlimited source for the research into transplantation treatment of this devastating disease.

Conclusion

Although initially the major goal of the establishment of hESC lines was the development of the cell-replacement therapies, it is presently obvious that hESC lines will have an important role in the studies of mechanisms of genetic disorders through generating the sources of normal and genetically abnormal cells and tissues. The ability to obtain hESC lines with specific genetic disorders, that could produce unlimited quantities of the disease tissue where the disease has a genetic basis, makes it realistic to undertake research on the primary disturbances of the cellular processes in the genetically abnormal cells and to identify the molecular mechanisms that might be

blocked to prevent the disease progression. Therefore, there is obvious need for establishment of hESC lines originating from embryos with genetic and chromosomal abnormalities, to provide the basis for understanding of the mechanisms of phenotype realization of genetic defects and for the development of new approaches for their possible treatment.

Our repository has a large collection of hESC lines, which provides a unique opportunity to screen available hESC lines for polymorphisms associated with susceptibility and/or resistance to diseases in humans, as demonstrated by the establishment of the first such line containing CCR5-32bp deletion, conferring resistance to HIV. So this approach may be productive for finding hESC lines with rare mutations which may prove invaluable to the future stem cell therapy of severe disorders for which there is no available treatment. This may have particular potential for research into the mechanisms of predisposition or resistance to common disorders, the results of which could lead to new treatment regiments to those common conditions for which there is no available treatment.

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Part IV

Impact of Stem Cell on Growth and Development of Human Fetus

Stem Cells in Growth and Development of the Human Fetus

20

Phuc Van Pham

Stem Cells

Stem cells are considered the origins of all organisms. In fact, all organisms are formed and developed from a single cell – the zygote, which is the product of oocyte fertilization by the sperm. This cell is referred to as a totipotent stem cell. Through the process of cell division, the totipotent stem cell produces all tissue types within an organism.

Stem cells have two important characteristics; the ability to undergo cell differentiation and self-renewal [5, 24]. Differentiation is a process by which a stem cell can become a functional cell. For example, hematopoietic stem cells (HSCs) can differentiate into red blood cells [8]. HSCs cannot transfer oxygen unlike their red blood cell decedents whose primary role is to carry oxygen. Self-renewal is a complex biological system which enables stem cells to maintain as well as increase their number. Stem cells perform self-renewal via asymmetric or symmetric division to ensure that at least one stem cell is produced from the cell division process.

By means of self-renewal, stem cells are maintained during embryonic development right through to adulthood. However, there are significant differences in stem cell properties between these stages. Therefore, stem cells are divided into at least two groups, including embryonic stem cells (ECSs) derived from the blastocyst and adult stem cells derived from the fetus or adult [5]. The main difference between them is that ESCs exhibit pluripotency, while adult stem cells only exhibit multipotency. Differences in the potential of these stem cells are summarized in Table 20.1.

In 2006, Yamanaka et al. (2006) showed that fibroblasts can be reprogrammed into pluripotent stem cells, and called these cells induced pluripotent stem cells (iPSCs) [49]. From this study, almost all mature cells or adult stem cells can be reprogrammed into iPSCs. iPSCs exhibit almost all ESC properties, including expression of pluripotent genes, such as Oct-3/4 and Nanog, multiple lineage differentiation, and successful production of chimeric mice. These cells offer much promise within biology and medicine. With respect to biology, studies of these cells have confirmed that all cells in the human body originate from a single cell, and that the offspring cells are different because of differences in their gene expression profiles. With respect to medicine, iPSCs open up a whole new therapy approach for the treatment of many chronic diseases [3, 44].

Another type of stem cell of particular interest in the field of cancer research is the cancer

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Table 20.1 The differences in potential of stem cells

Potential	Number of differentiated cells	For example	Types of differentiated cells
Totipotent	All	Zygote (or Blastomere)	All cells in human body and extra-embryonic parts
Pluripotent	All, exclude trophoblast	All cells from inner cell mass	All cells from three germinal layers
Multipotent	Many	Hematopoietic stem cells	Cardiac cells; muscle cells; osteoblasts; hepatic cells; all kinds of blood cells
Oligopotent	Some	Myeloid	Five kinds of blood cells: red blood cells; monocytes; macrophages; eosinophils; neutrophils
Quadripotent	Four	Mesenchymal progenitor cells	Chondrocytes; adipocytes; glial cells; osteoblasts
Tripotent	Three	Glial progenitor cells	Two kinds of astrocytes; and oligo-dendrocytes
Bipotent	Two	Mouse liver derived precursor cells	Lymphocyte B and macrophage
Unipotent	One	Mast cell precursor	Mast cells

stem cell. Cancer stem cells are defined as the original cells giving rise to malignant tumors [6, 47]. To date, the origin of cancer stem cells is hypothesized differently; with the most accepted origin of the cancer stem cell being a mutated stem cell. When normal stem cells become mutated, especially with respect to their self-renewal-related genes, they can proliferate uncontrollably and thus form tumors [51].

In general, stem cells play an extremely important role in the formation, growth and development of all organisms. In this chapter, the role of stem cells in the growth and development of the human fetus is demonstrated.

Early Embryo Development and the Origin of Stem Cells

To date, there has been much debate about the existence of stem cells in the early embryo. Some authors suggest that in fact no stem cell exists in the early embryo and that at this stage there are complex changes in the development of embryonic cells, such as multiplication, commitment, differentiation, and death.

For example, the unfertilized oocyte may be regarded as a pluripotent cell, which can be activated to develop into the embryo in a parthenogenic manner [46]. Although ESCs can be successfully isolated from these embryos, results in primates and mice have shown that they cannot grow and develop into whole organisms [17, 26, 52]. This means oocytes cannot be regarded as totipotent cells, while zygotes are totipotent stem cells because they can develop into whole organisms. Results from embryo splitting technology show that only the zygote and blastomere at the eight-cell stage embryo are in fact totipotent [38, 43].

In the next stage of embryonic development, differentiation of totipotent stem cells starts to form at least two cell types within the blastocyst, including trophoblasts and the inner cell mass. Cells in the inner cell mass maintain the stemness with pluripotency and are so-called ESCs [2, 48]. Conversely, trophoblasts are differentiated cells that form the extraembryonic tissues, such as the placenta. This is the first commitment event in mammalian development. When ESCs loose the capacity to produce trophoblasts, they become pluripotent not totipotent stem cells.

The next commitment event relates to the formation of the primitive endoderm, the precursor of the yolk sac. The yolk sac can be considered a primitive form of the placenta, which plays the vital role in the transport of nutrients from the mother to fetus.

The inner cell mass comprising ESCs continues to develop and form an epithelium, known as

the epiblast. The epiblast continues to maintain pluripotency with the ability to differentiate into multiple tissues, as well as form teratomas when transplanted into ectopic sites. In fact, ESCs can also be isolated from the epiblast [10, 12, 25]. However, there is a difference between the inner cell mass-derived ESCs, which can integrate into a host blastocyst to form a chimera, and epiblast-derived ESCs, which loose this potential. In the next stage of embryonic development, the epiblast progresses to the gastrulation stage, with the formation of three germinal layers. The cells in these three germinal layers become more specific, thus loosing pluripotency. In fact, during the process of gastrulation, the intensities of Wnt, nodal and BMP signaling determine cell fate in the epiblast. Development from gastrulation to the fetus is a complex process that results in the formation of all specific tissues and organs. It is believed that gastrulation-specific stem cells are produced by self-renewal of the epiblast.

Roles of Tissue Specific Stem Cells in Morphogenesis: Breast Model

The mammary gland is a specific organ in the human body that continues to undergo morphogenesis postnatally. Therefore, mammary gland morphogenesis has been studied in-depth. In fact, the mammary gland changes with respect to both tissue structures as well as cell population during puberty, pregnancy and menopause. To date, many studies have confirmed that all these processes are driven by mammary stem cells. Early evidence has shown that normal human mammary epithelium is developed from mammary stem cells, a process first observed through studies of the X-chromosome inactivation pattern. In 1996, Tsai et al. analyzed cells from normal mammary epithelium and showed that all cells had the same pattern of X-chromosome inactivation [50]. This means that all cells in the same mammary gland originate from a single cell.

In the mouse model, one single mammary stem cell can regenerate the entire mammary gland *in vivo* [35, 36]. In humans, xenotransplantation using human mammary stem cells injected into the mammary fat pad or under the renal capsule of immune-deficient mice *in vivo*, results in outgrowths equivalent in size to one human mammary lobule.

Further investigations have enabled normal human mammary stem cells to be characterized based on specific marker expression and differentiation potential. As a stem cell, human mammary stem cells exhibit prolonged self-renewal; differentiate along both luminal and myoepithelial lineages, undergo branching morphogenesis in three dimensional culture, and generate outgrowths in xenotransplantation experiments. From the mammary cell population, mammary stem cells can be enriched by surface markers including $CD49f^{high}EpCAM^{low}$ [9], $CD73^{+}CD90^{-}$ [40], $CD10^{+}$ [23], and $CD49f^{+}DLL1^{+}DNER^{+}$ [34]. Mammary stem cells can also be enriched based on expression of high expression of aldehyde dehydrogenase (ALDH) [13]; or based on mammosphere culture [34].

Human mammary development starts from 4 to 6 months of embryonic development [18]. At this time point, the primary mammary gland contains a central and peripheral basal cell population that produces two different cell layers [22]. From 21 to 25 weeks, epithelial buds form from the primary bud. In some cases, branched ducts highly structured together with regular lobules form similar to those observed in adults [31, 41] (Fig. 20.1).

Neural Stem Cells: Generating the Brain

Formation of the CNS starts with neural plate formation, followed by neural tube formation, which comprises one layer of neuro-epithelial cells. After the neural tube matures, the cellular architecture becomes stratified and neural stem cells are found in the ventricular layer, while the post-mitotic cells migrate toward the brain surface [1]. Cortical development is the result of symmetric cell division of neural stem cells. Asymmetric cell division produces two distinct cell types: one stem cell and one immature neuron or intermediate progenitor cell (IPC) (also called a basic progenitor). Immature

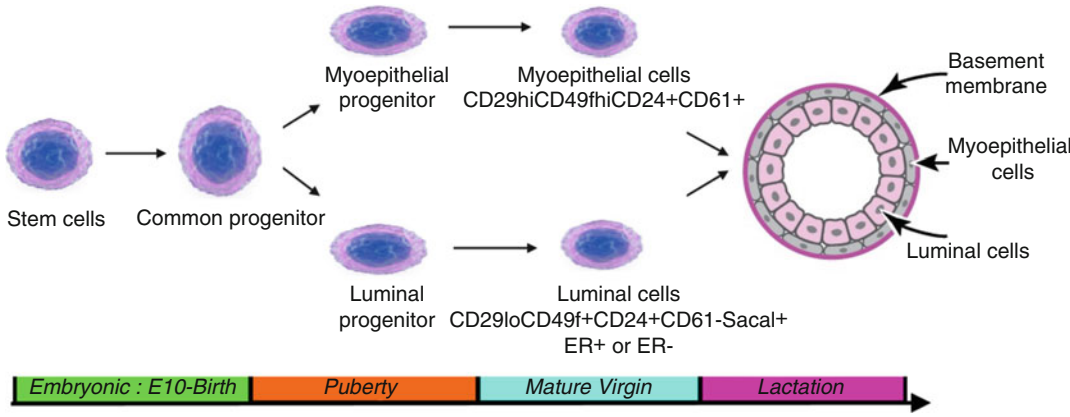

Fig. 20.1 Stem cells and mammary development. Mammary gland is formed by mammary stem cells. These cells can differentiate into two kinds of mammary gland included myoepithelial cells and luminal cells

neurons migrate away from the ventricular zone and become mature neurons of the cortical plate, whereas the intermediate progenitors reside in the subventricular zone, where they continue to divide and constitute an important reservoir for new neurons throughout neurogenesis. The intermediate progenitors are also able to divide symmetrically, generating two progenitors or two neurons [16, 30].

Neuroepithelial cells are gradually replaced by radial glia [27]. These radial glial cells express several astroglial properties and markers specific to the glial lineage, such as RC2 [29]. Radial glia can divide asymmetrically and serve as progenitors of neurons and glia, as well as constitute a scaffold onto which neurons migrate in the developing brain. Furthermore, it is now appreciated that they are in fact stem cells and similar to neuroepithelial cells. However, radial glial cells have a more restricted potential than neuroepithelial cells. *In vivo* evidence of neuroepithelial cell tripotency has been demonstrated by retroviral trace labeling, whereas the majority of labeled radial glia are found to give rise to a single cell type, for example a neuron, astrocyte, or oligodendrocyte [15, 28] (Fig. 20.2).

Hematopoietic Stem Cells and Their Roles in Blood Formation

In humans, HSCs originate from the mesoderm/hemangioblast. From day 21, hemangioblasts mature into pre-HSCs and HSCs first appear in the AGM (aorta-gonad-mesonephros) region from day 28 [14]. They continue to colonize the developing fetal liver, therefore the fetal liver becomes the main source of HSCs until birth, at which point bone marrow hematopoiesis is established. However, there are two regions related to HSC production including the AGM and yolk sac.

A long-standing hypothesis posits that hematopoietic cells originate from the hemangioblast – a mesodermal precursor cell that gives rise to blood and endothelial cells. These precursors develop between days 2.5 and 4.0 of embryoid body (EB) differentiation and form blast-like colonies (referred to as blast colony forming cells, BL-CFCs). These precursors give rise to primitive and definitive hematopoietic, endothelial and vascular smooth muscle cells (Fig. 20.3).

BL-CFCs have been isolated from brachyury$^+$/Flk1$^+$/Kdr$^+$ (Kdr: kinase insert domain protein receptor) cells, indicating that they are a specialized subset of the mesoderm [11]. Their subsequent commitment into the hematopoietic fate is driven and marked by expression of Scl/Tal1 (T-cell acute lymphocytic leukemia 1) [7, 37]. More importantly, Flk1$^+$brachyury$^+$ hemangioblasts can also be detected in the gastrulating mouse embryo [19]. However, surprisingly few of the BL-CFCs that display both hematopoietic and vascular potential are found in the yolk sac.

Hemangioblasts are suggested to be precursors of yolk sac hematopoietic cells. This implies

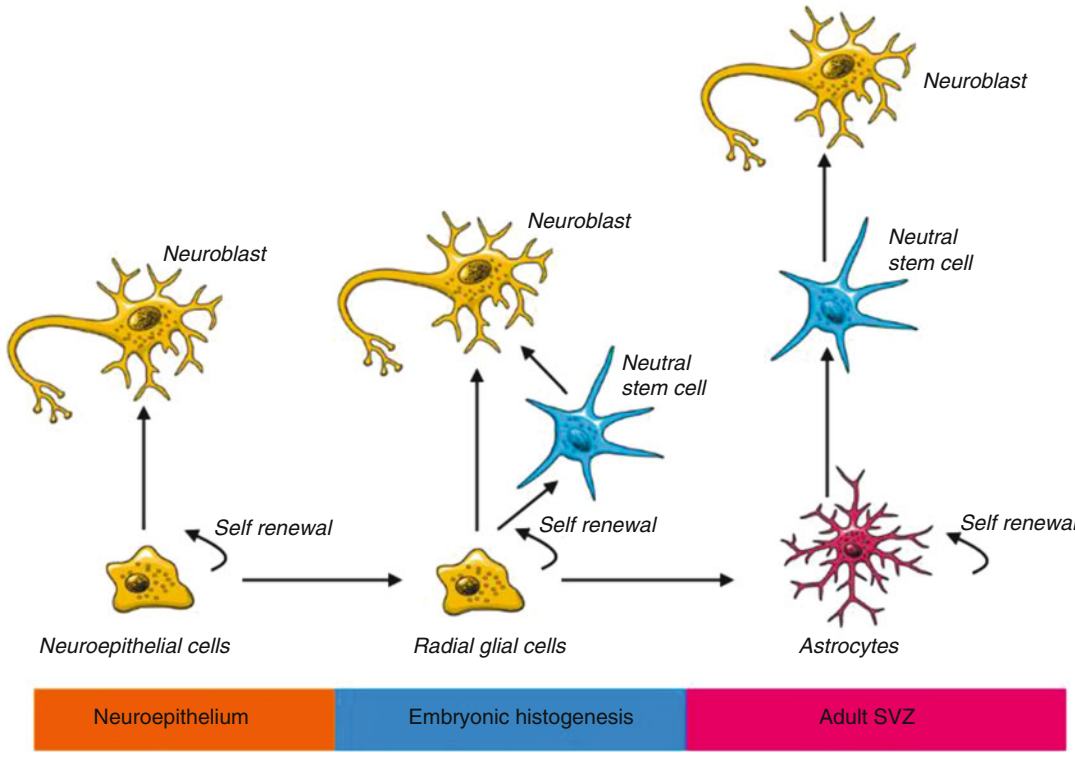

Fig. 20.2 Neural stem cells (NSCs) in development and in the adult. Neuroepithelial cells in early development divide symmetrically to generate more neuroepithelial cells. Some neuroepithelial cells likely generate early neurons. As the developing brain epithelium thickens, neuroepithelial cells elongate and convert into radial glial cells. Radial glial cells divide asymmetrically to generate neurons directly or indirectly through neural stem cells. Oligodendrocytes are also derived from radial glial cells through neural stem cells that generate oligodendrocytes

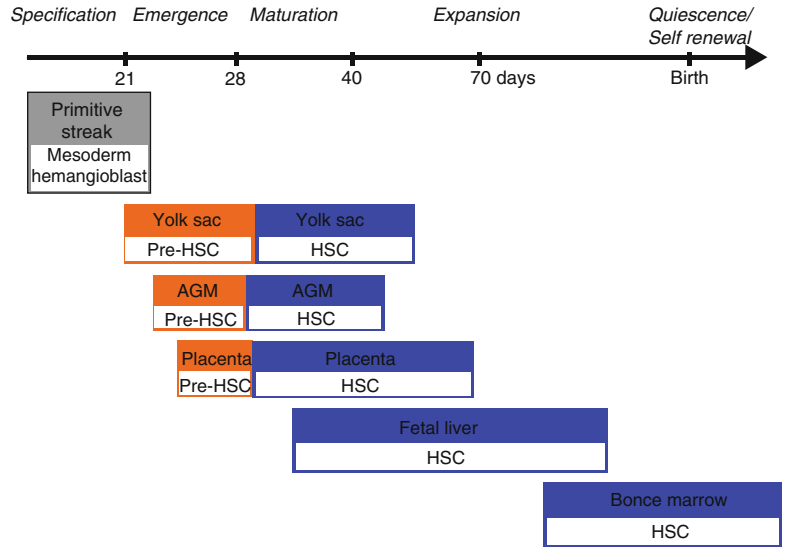

Fig. 20.3 Establishment of definitive hematopoietic stem cell (*HSC*) pools in human. Hematopoietic development starts as specification of primitive streak mesoderm into hematopoietic and vascular fates. Pre-HSCs at yolk sac, AGM or placenta would go to a maturation process to produce HSCs in yolk sac, AGM, placenta, fetal liver and bone marrow. Subsequently, fetal HSCs expand rapidly, after which a steady state is established in which HSCs reside in a relatively quiescent state in the bone marrow

that the first stages of hematopoietic development take place before the cells migrate into the yolk sac, prior to the formation of blood islands, which consist of developing primitive red cells and endothelial cells adjacent to the visceral endoderm [33].

Migration of hematopoietic precursors from the primitive streak to the yolk sac depends on vascular endothelial growth factor signaling through Flk1 [45]. Further studies have shown that specification of hematopoietic fate in the yolk sac also depends on the visceral endoderm and on signals from Ihh (Indian hedgehog) [4] and Bmp4 (bone morphogenetic protein 4) [42], demonstrating the importance of the microenvironment for hematopoietic commitment.

Errors of Stem Cell Self-Renewal, Differentiation and Diseases

Fetal Alcohol Syndrome and Neural Stem Cells

Fetal alcohol syndrome (FAS) is considered the result of the effect of alcohol on neural development of the fetus, which interferes with differentiation and the formation of neurons and glial cells from neural stem cells. FAS usually occurs in children born to mothers who have abused alcohol during pregnancy. Some FAS defects include pre- and post-natal growth deficiencies, minor facial abnormalities, and damage to the developing central nervous system (CNS). Many FAS-related defects occur in the CNS, with gross morphological abnormalities of the brain and when compared to normal children, an overall decrease in white matter, particularly in the cerebrum or forebrain. Certain areas of the developing CNS are particularly susceptible to alcohol-induced birth defects, including the ocular system, corpus callosum, basal ganglia, and cerebellum. From 5 to 6 weeks after fertilization, alcohol also impacts the corpus callosums of fetuses. The corpus callosum is a dense band of white matter that separates the right and left hemispheres of the brain and is responsible for coordinating communication between hemispheres. Corpus callosum defects can lead to poor bimanual motor coordination or motor-visual coordination, and issues with faculties like abstract thought and decision making.

Alcohol is also considered as an apoptotic agent that causes damage to neural stem cells or neural progenitor cells, like radial glia, which give rise to neurons and supporting glial cells in the CNS. Radial glia not only provide a physical scaffolding but also chemically guide neurons as they form. Alcohol can impact the development and migration of these radial glia progenitor pools, thereby resulting in fewer neurons and glia being produced. In particular, neurons as well as glia with morphological abnormalities can be produced. These effects reduce cell volumes and cause structural abnormalities, which can in turn impact the CNS.

Myelodysplastic Syndrome (MDS): A Stem Cell Disorder

Myelodysplastic syndrome (MDS) comprises a group of diverse bone marrow disorders in which the bone marrow does not produce enough healthy blood cells. MDS is often referred to as a "bone marrow failure disorder". MDS is primarily a disease of the elderly (most patients are older than 65 years), but MDS can affect younger patients too. There are three or four phases of MDS, including progression to acute leukemia, and the loss of proliferation or differentiation capability, leading to progressive cytopenias. Cytopenias are low blood cell counts, and are a hallmark feature of MDS responsible for some of the symptoms that MDS patients experience, including infection, anemia, spontaneous bleeding, or easy bruising. In addition to reduced numbers of blood cells, the mature blood cells circulating in the blood system may not function properly because of dysplasia. Failure of the bone marrow to produce mature healthy cells is a gradual process, and therefore MDS is not necessarily a terminal disease.

To date, a number of studies have confirmed that MDS is related to defects in HSCs. These defects give rise to secondary stem cell abnormalities,

for example involving stem cell niche interactions or immune function. The MDS patient HSC pool has been shown to undergo increased apoptosis as well as decreased proliferation and survival. HSCs up-regulate a number of interferon (IFN)-inducible genes. Depletion of early HSCs is seen in MDS, suggestive of defective self-renewal. This means there are some errors in the genes regulating the self-renewal, differentiation and quiescence of HSCs in MDS patients. The stem cell niche is a micro-environment providing protection to HSCs, especially in keeping HSCs quiescent and protecting them from oxidative stress. Therefore, defects in signaling between stem cells and their niche also cause depletion of HSCs.

Auto-immune Diseases as Stem Cell Disorders

Using animal models, a number of studies have found that autoimmune diseases are actually stem cell disorders. Interestingly, HSC transplantation can be used to treat these diseases; however transplantation of abnormal HSCs from autoimmune disease mice to normal mice may trigger autoimmune disease in the recipients. Some researchers have successfully identified and cultured both normal and abnormal HSCs and identified some of the differences between them.

In an early study, Morton and Siegel transplanted bone-marrow cells from lupus-prone NZB mice into lethally irradiated non-susceptible strains and induced lupus syndrome [32]. In another study using the same method, bone marrow transplantation transferred the autoimmune diseases systemic lupus erythematosus (SLE), experimental autoimmune encephalomyelitis (EAE), adjuvant arthritis, antiphospholipid syndrome and type 1 diabetes, into the recipients [20].

It is interesting that bone marrow transplantation, also called HSC transplantation, is reported to be the best way to treat many autoimmune diseases. For example, in a report of more than 195 methods used to prevent or delay type 1 diabetes in non-obese diabetic (NOD) mice, HSC transplantation was highlighted as the most effective method [39]. Allo-transplantation of HSCs also was considered as a suitable and effective therapy in treating SLE [21].

Conclusions

Origins of stem cells in human fetal development and growth are unclear. Stem cells exist in the early embryo, including the blastocyst, and in the adult, as confirmed during gastrulation and fetal development stages. Furthermore, adult stem cells can be produced from ESCs. Theoretically, all adult stem cells originate from ESCs via the process of self-renewal and differentiation. However, to date, gaps remain between the science of stem cells and that of human development. Some scientists believe that adult stem cells are formed early during fetal formation, however different terminologies exist between development and stem cell biologists regarding these cell types. It is therefore essential to unify these different terms in order to clarify stem cell roles and formation. Despite this, stem cells clearly exhibit important roles during fetal development and growth. A greater number of stem cell disorders are currently being discovered and the effects of stem cell damage on normal fetal development are gradually being elucidated.

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The Mystery of the Survival of the Human Fetus in Utero

21

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Introduction

The study of the body's immunity and immune defense system has enabled physicians to understand how in a successful pregnancy, the baby is allowed to grow inside the mother's body without being rejected. All healthy individuals have an immune system that will reject germs, viruses and donated organs, e.g., kidneys, hearts and livers, with the immune rejection reaction. However, during a successful pregnancy, this outside interference with the mother's immune system in not activated.

During a successful pregnancy, the immunity that may cause rejection of the baby and placenta is shut off in the mother's uterus. Throughout the rest of the mother's body, her immune system is fully functional, allowing her to deal in a proper way with any infections that may come her way. Unfortunately many couples with recurrent spontaneous miscarriages find themselves in a situation where, following conception, this immune camouflage and protection of the embryo is not initiated in such a way that the uterine local and systemic immune responses of the mother are modulated in favour of a decrease in cell mediated immunity and increase in humoral immunity [1].

Discussion

Scientists have understood for years how to manipulate and interfere with the immune system and its functioning and the prevention of Rh sensitization during pregnancy, hemolytic disease of the newborn being a clear example. In this case, the injection of readymade Rh immunoglobulin (Rh antibody) in to the Rh negative mother carrying an Rh positive baby prevents her own immune system from recognizing and rejecting the baby's Rh positive cells. Briefly, the mother-to-be receives signals (molecular messages) from the conceptus. Many of these messages are hormonal (endocrine) in nature; however, others are direct genetic messages that the father contributed to the child. Some of these involve the tissue type (HLA antigens) that the father gives to the child. If the parents are appropriately "mis-matched", so far as their tissue types are concerned, the mother's system recognizes that the baby's cells

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(the trophoblasts) that will form the placenta are "foreign". The lymphocytes of her immune system that have congregated in her uterus communicate with the molecular messages from the baby's cells and begin to protect the baby from immune rejection. The mother's immune system forms an antibody that can be measured as early as 5 weeks of pregnancy. This antibody attaches to the molecules on the cells of the baby that induced them. Here, they camouflage the baby's cells from the mother's immune killer cells that can destroy. In addition, the attachment of this specific antibody to the baby's cells brings with it a signal that makes them grow and divide [2]. Without this "growth" signal, the baby and the placenta regress and die.

If the maternal antibody which leads to the protection of the baby is low, the baby is not effectively shielded and protected. The cells of the placenta are not stimulated to grow, because there is no antibody produced by the mother. The outcome over the next few days or weeks will be the rejection of the fetus. By immunological testing and HLA tissue typing, these couples look more like brother and sister than unrelated husband and wife. In sum, the message from the father communicated to the mother via the tiny conceptus is not heard by the mother. Immunoprotection is not induced and the pregnancy fails repeatedly [3].

The problems of "lucky" or "unlucky" match can be addressed together through tissue typing and identifying the HLA antigens of both. In addition, the couple's ability to respond appropriately to each other can be tested through a laboratory assay called the mixed lymphocyte culture reaction.

Another problem that interferes with a subsequent pregnancy success operates through an alteration of the blood clotting mechanisms. Each pregnancy which the mother loses, is associated with an approximately 20 % increased chance that she will make an autoimmune response to fatty molecules called phospholipids that are integral parts of the baby's cells. There is a family of phospholipids: the one best known and most tested for is cardiolipin—the heart fat. Cardiolipin or one of its 'brothers' and "sisters" in the mother, may activate and speed up her blood clotting in the vicinity of early pregnancy and this results in its loss. Conclusions in pregnancy may occur later in gestation such as intrauterine growth retardation, toxemia and even intrauterine fetal demise. Half of these will be premature and the other half will have features of growth retardation, the remainder of the babies will possibly die during pregnancy due to abnormalities of the anti-phospholipid antibody and a demonstrated clotting abnormality (i.e., the lupus anti coagulant) [4].

Other causes of recurrent spontaneous abortion include hormonal problems, abnormal development or diseases of the uterus or cervix, poor sperm quality, infections, chromosome abnormalities and chronic diseases.

Every person's white blood cell type (tissue type, HLA type) consists of ten numbers, half of which comes from each parent. These white blood cell numbers are molecules (antennae) that are present on the surface of the person's white blood cells, and they serve important functions in recognizing foreign antigens (germs and viruses) that enter our body. This recognition even results in an immunity response that eventually leads to death of the germ or virus and the formation of an antibody against the germ or virus that prevents recurrence of the disease.

A representative white blood cell type is as follows: A1.2, B7.12, C4.2, DR 3.8 and DQ 4.1. One number at each locus (A, B, C, DR and DQ) comes from the mother and the other from the father.

Couples with secondary abortions (one living child and then spontaneous miscarriages) are a distinct group. Sixty percent of the first-born children of secondary couples are DQ 0501/501 (formally called DQ 4.1 homozygous). This means that they are purebred with regard to this part of their white blood cell type. Both mother and father contributed the same 4.1 gene. An analysis of several hundred live born children who were born following immunological treatment of their mothers revealed no live born DQ 0501/0501 babies. Women whose first babies are DQ 0501/0501 are at high risk of making an autoimmunity response that involves the production of an antibody (immunity) in their body that attacks the glue (phospholipids) that are impor-

tant in building the placenta of the baby. They are also prone to increase the numbers of natural killer cells in their blood from 4 % to over 20–30 % [3].

Further studies in the laboratory of Prof. Alan Beer in 1995 showed that this type of natural killer cells in women with recurrent miscarriage can also damage the early cells that will eventually make the placenta. (Beer AE. Personal communication on the problem of abortion with intra-amniotic antigen on 09.05.1997.)

Now, thanks to a better understanding because of contributions from AE Beerr, AM Bahar et al., JYH Kwak et al. [5–6] on immunological factors that protect or destroy the developing embryo and fetus, researchers using simple new therapies are reporting very high rates of successful pregnancies among thousands of women who have experienced previously unexplained recurrent miscarriages [7–10].

The treatments are designed to counter one or two types of immunological problems that can result in destruction of the placenta, embryo or fetus by antibodies produced by the pregnant woman. One approach is designed to block antibodies against the fetus by the mother's own tissues. In the language of Prf. Beer, 'Blocking antibodies create a disguise and the fetus becomes a wolf in sheep's clothing as far as the mother's immune system is concerned' [3, 11, 12].

Now, with a better understanding of the pathophysiology of pregnancy we can see that highly specific, protecting systems operate during pregnancies which do not interfere with any of the mother's immune responses. Immunoglobins (IgG) are available commercially. If a pregnant woman were to receive a kidney from an unmatched donor, she would reject it at any other time. Similarly, her body can continue to fight off outside infectious agents [13–16] but not the pregnancy due to the presence of this blocking antibody in the maternal system.

Since half the genetic information in the fetus comes from a "foreigner", namely the father, without blocking antibodies acting as an immunological fortress, the mother's body would launch an attack against it. But nature does not rely on the mother alone to protect the fetus. As indicated by another set of studies, the substance that stimulates the production of blocking antibodies comes from the father, carried in by the sperm that produces the pregnancy [17].

Dr. JYH Kwak et al. [18] and Prof AE Beer et al. [18] have however, indicated that sometimes the tissue of the father and the mother are too much alike immunologically and the woman's body fails to respond adequately to the father's feeble signal [19] to produce blocking antibodies. This results in outright rejection of the pregnancy tissue or failure of the fetus to thrive.

Normally, the signal to produce blocking antibodies comes from the paternal contribution to the embryo. The antibodies can be measured as early as 5 weeks of pregnancy. But if the maternal and paternal tissue types are too similar the signal is not received and the pregnant woman does not produce the protective antibody [20].

Allopregnancy Is a Th2-Like Phenomenon

According to one study, CBA × DBA/2 mice are particularly susceptible to low doses of Th1 mediators (TNF, gamma IFN), which act in synergy to induce high fetal loss. In addition, CBA × DBA/2 placentae and deciduae produce few, if any, Th2 cytokines such as IL-4, IL-3 and IL-10. Alloimmunisation, which is known to prevent resorptions in this abortion-susceptible combination and to prevent TNF- or LPS-induced abortions/resorptions, enhances the placental production of IL-4 and IL-10 in CBA × DBA/2 matings. Furthermore, rIl-10 given alone by intraperitoneal injection completely reverses the high incidence of fetal resorptions, in control to anti IL-10. Anti-gamma IFN and pentoxifylline (an anti-TFN agent), which partially reduce resorption, act in synergy for optimal fetal protection. Injection of recombinant bovine trophoblast protein (r.o TP) corrects the high rate of fetal resorption in CBA × DBA/2 mice and is correlated with increased placental IL-4 and IL-10 production. These results show that Th2 play a more important role than Th1 and suggest that a simple immune-endocrine network is involved in

establishing the characteristic Th1/Th2 balance observed during pregnancy [21].

The results of many studies indicates that non-specific killer cells and inflammatory, cytostatic and cytotoxic lymphokines play a crucial role in immunologically mediated fetal death in both naturally abortion-prone CBA×DBA/2 and B10×B10. A mice have a higher resorptions rate, which increases with age and the prevalence of "bad father" [22]. A correlation has been shown between these high resorptions rates and accumulation of asialo GM1+cells at the implantation sites of the embryos that are to be resorbed [23]. Activation of these cells by poly-IC or gamma-IFN lead to abortion [1, 24, 25].

Role of TJ6 Protein during Pregnancy

TJ6 is a novel immune suppressor protein which can induce apoptosis.

Early studies demonstrated that mice treated with antibody to the TJ6 protein early in pregnancy resulted in ablation of those pregnancies. The gene for TJ6 was subsequently cloned in both man and mice and were found to be nearly identical. Antibodies and DNA probes prepared from one species are reactive with the other. Expression appears to be highly regulated and in part (1) Cell activation and (2) Steroid hormone. Other factors such as mitogens and cytokines appear to modulate these effects.

Flow cytometric analysis of TJ6 on peripheral blood lymphocytes of pregnant women revealed that TJ6 was expressed on CD19 positive B cells. Specifically, TJ6 is expressed as a 45 kD membrane form on the surface of these cells. While the initial protein was shown to be approximately 70 kD in length, two additional forms have been identified. Subsequent studies show that the TJ6 is post-translationally modified to produce a 45 kD membrane form and an approximately 18 kD soluble form. Some preliminary studies have shown that the membrane form is involved in programmed cell death of the cells expressing it. The soluble form appears to be involved in antiproliferative effects of anti-CD3 and allogeneic stimulated cells [26].

Reproductive Immunophenotype

If we study in detail the reproductive phenotype which is essential for its manipulative role in the continuation of pregnancy, it is seen that there are 30 different types of white blood cells (Lymphocytes). However, eight of them are most important and can be assessed by flow cytometry. Disorder of these eight cell types may predict a future pregnancy loss. These tests were developed by Professor Alan Beer. (Personal communication and suggestions of Prof. Alan E. Beer on the problem of abortions with intra amniotic antigens on 09/05/1997.)

A. CD3 (Pan T cells) range between 63 % and 86 %. They are low when the immune system is suppressed. They are high when the immune system is overactive.
B. CD4 (T Helper cells) range 31–53 %. They are destroyed by HIV virus. If they are low the aetiology of the deficiency must be studied.
C. CD5 (T cytotoxic suppressors) range 17–35 %. They coordinate the strong or weak immune reaction by coordinating Pan T and helper T cells.
D. CD19 (B cells) range 3–8 %. These lymphocytes are plasma cells that produce antibody 19 m in the blood, then IgG in the lymph system and lastly, the IgA protects the organ (organ immunity). CD19 is high in cases of women with an immune related cause for infertility.
E. CD56+CD16+Natural killer cells, range 3–12 %. These are natural killer cells produced in the bone marrow and produce tumor necrotic factor. If level varies above 18 %, intravenous immunoglobin may help the situation, otherwise may result in reproductive failure.
F. CD56+Natural killer cells, range 3–12 %. They lack CD16 molecules or rather, known as CD56+CD16 natural killer cells, which could be identified in decidua and may also

produce large quantity of tumor necrotic factor in the decidua and can kill placental cells or the fetal cells. A level of 18 % or above predicts poor pregnancy outcome and may necessitate I.V. Immunoglobin G therapy which can decrease the killing potential of the natural killer (NK) cells.

G. CD3 IL-2 R cells, normal range 0–5 %. They are high in autoimmune disease in case of rejection of kidney or bone graft. If the level is above 10 % I.V. immunoglobin may prevent its activation.

H. CD19 + CD5+ (B1 cells), range 0–3 %. When activated they produce antibody against hormone, hormone receptor and neurotransmitters and may also justify autoimmune condition or rejection of an organ, e.g., bone marrow.

Conclusion

Why a pregnancy survives, remains an eternal mystery. There are many concepts and working hypotheses, that try to explain why the fetus survives in utero. Many factors, notably complement's role, chromosomal role of the parents, steroid and immunosuppressive role, manipulation by immunophenotypes, novel immunosuppressor's role etc have been highlighted in many research papers over the last 50 years. Yet, none of these explanations can comprehensively explain the survival of the human fetus.

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Part V

Fetal Endocrine Development up to Second Trimester

Glucose Metabolism in Foetus and Its Relationship with Foetal Insulin

22

Prabir Kumar Kundu

Introduction

Glucose is the principal blood sugar of the human foetus and its main energy source. Not surprisingly, therefore, its supply to these tissues is regulated by a relatively complex set of mechanisms that tend to keep its metabolism relatively constant [1]. The first point in this regulation is the maintenance of maternal glucose concentration by increasing rates of maternal glucose production and development of relative maternal glucose intolerance and insulin resistance. The second point is the transfer of maternal glucose to the fetus by the placenta, which is buffered by placental glucose utilization. The third point is the production of insulin by the developing foetal pancreas, which enhances glucose utilization among the insulin-sensitive tissues (skeletal muscle, liver, heart, adipose tissue) that increase in mass and thus glucose need during late gestation [2, 3]. Glucose uptake into foetal tissues is regulated by glucose transporters that increase or decrease in response to both acute and chronic changes in foetal glucose concentration and conditions of intrauterine growth restriction (IUGR). At the same time, signal transduction protein regulators of amino acid synthesis into protein are down-regulated, emphasizing that IUGR presents a mixed phenotype, with increased propensity to take up energy substrates, such as glucose, and diminished capacity for protein synthesis and growth [4].

Knowledge and understanding of the foetal glucose understanding is necessary to understand the nutritional requirements for normal growth and development, to provide rationale treatment for acute foetal distress and to provide prenatal care of a diabetic patient. Foetal glucose metabolism depends on additive effects of foetal plasma glucose and insulin. Glucose-stimulated insulin secretion increases over gestation, is down-regulated by constant hyperglycemia, but enhanced by pulsatile hyperglycemia. Insulin production is diminished in fetuses with intrauterine growth restriction (IUGR) by inhibition of pancreatic cell replication, but not by mechanisms that regulate insulin production or secretion, while the opposite occurs with hypoglycemia alone, despite its common occurrence in IUGR. Chronic hyperglycemia down-regulates glucose tolerance and insulin sensitivity with decreased expression of skeletal muscle and hepatic Glut 1 and 4 glucose transporters, while chronic hypoglycemia up-regulates these transporters. The opposite occurs for signal transduction proteins that regulate amino acid synthesis into protein. Such adaptations might underlie childhood and adult metabolic disorders of insulin resistance, obesity, and diabetes mellitus.

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Glucose Homeostasis Regulation in the Foetus

The foetus is constantly supplied with glucose that passes through the placenta. Glucose, amino-acids and lactate are the main energetic substrates during the foetal development. Glucose is the source of approximately half of the total energy necessary for the growth and development of the foetus. It passes through the placenta by diffusion and the amount that is being used up by the foetus depends on the mother's blood glucose level as well as on the transplacental glucose gradient. Foetal blood glucose level comprises approximately 70–80 % of the glucose concentration in the mother's venous blood serum. The foetus, besides taking the glucose from the maternal blood, utilizes also quite high amounts of amino acids and in this way the mother is being deprived of gluconeogenesis precursors. For this reason pregnant women develop hypoglycaemia faster than before pregnancy. Foetal metabolism is set on anabolic processes. This is the reason for building up glycogen, which is stored in the liver during late pregnancy and is being used as an easily accessible source of glucose in the first hours after birth. Enzymatic systems necessary for gluconeogenesis and glycogenolysis are present in the foetal liver, but remain inactive and are activated only in case of extreme maternal starvation. The foetal liver contains approximately three times more glycogen than the liver of an adult and during birth this amount constitutes the energetic reserve for the newborn. Fat oxidation plays during foetal live a less important role than the oxidation of aminoacids and glucose, so the production of ketones is low. Insulin, similarly as in the other periods of life, is the main anabolic hormone of the foetus and can be detected in the foetal pancreas already in early development stages. Endocrine pancreatic cells develop between the further investigations. It seems however, that to protect the anabolic processes – glycogen and fat tissue formation – a high insulin/glucagon ratio is being maintained.

These observations underline the importance of the appropriate functional development of the endocrine part of the pancreas for the foetal development. Investigations concerning the amount of receptors and their hormonal affinity revealed, that these both parameters are decreased for glucagon receptors in contrary to insulin receptors, which number and insulin affinity are significantly higher than in an adult pancreas. This phenomenon also indicates a major predominance of anabolic processes in this part of life.

> Insulin does not pass through the placenta and therefore the foetus has to be independent from the mother. Insulin secretion in the foetus is regulated by the concentration of glucose as well as the concentration of amino acids, but the β cell of the foetal pancreas becomes with time more and more susceptible to glucose stimulation. A particularly intensive increase of β cell mass takes place in the last trimester of pregnancy. This development influences numerous other processes in the future life and may play a deciding role in the predisposition towards different diseases in adulthood. Young adults with a very low birth weight have higher indexes of insulin resistance and glucose intolerance and higher blood pressure than those born at term [6]. During this time the development of the exocrine part of the pancreas takes place and the islets begin to be formed (these structures are fully developed in the 22 gestation week). In comparison to adults, foetal β cells are characterized by decreased response to blood glucose and amino acids levels alterations in vivo as well as in vitro.

Foetal Endocrine Pancreas

The foetal pancreas appears during the fourth week of foetal life. The alpha cells, which contain glucagon, and the beta cells, which contain somatostatin, develop before the beta cells differentiate; however, insulin can be recognized in the developing pancreas before beta cell differentiation is apparent. Human pancreatic insulin and glucagon concentrations increase with advancing foetal age, and are higher than concentrations found in the adult pancreas. In vivo studies of umbilical cord blood obtained at delivery and foetal scalp blood samples obtained at term show that foetal insulin secretion is low and tends to be relatively unresponsive to acute changes in glucose. In contrast, foetal insulin secretion, *in vitro*, is responsive to amino acids

and glucagon as early as 14 weeks' gestation. In maternal diabetes mellitus, foetal islet cells undergo hypertrophy such that the rate of insulin secretion increases [9, 10].

- **Week 7 to 20 – pancreatic hormones secretion increases, small amount maternal insulin**
- **Week 10 – glucagon (alpha) differentiate first, somatostatin (delta), insulin (beta) cells differentiate, insulin secretion begins**
- **Week 15 – glucagon detectable in foetal plasma**

Foetal Insulin Secretion

The foetal pancreas develops in the late first to early second trimester, producing measurable insulin concentrations by mid-gestation. There is a gradual increase in basal insulin concentration and glucose- and arginine-induced insulin secretion towards term in foetal sheep [2].

Regulation of Insulin Secretion by Glucose Concentration

Foetal insulin secretion responds variably to changes in glucose concentration that are dependent on the absolute change in glucose concentration and its duration, magnitude and pattern [6]. For example, glucose stimulated- and basal insulin secretion in foetal sheep in late gestation are down-regulated in the presence of chronic, sustained, marked hyperglycaemia [5, 6] but pulsatile hyperglycaemia increases foetal insulin secretion, as occurs in human fetuses and neonates [6, 12]. Interestingly, hypoglycaemia also decreases basal and glucose-induced insulin secretion [16, 17], evidence of how intrauterine growth restriction, which characteristically among human clinical cases and animal models involves foetal hypoglycaemia, decreases foetal pancreatic development and insulin secretion capacity. Amino acids also regulate insulin secretion, but for the most part, acute and very high physiological to pharmacological changes in a given amino acid's concentration are necessary to elicit a significant change in foetal insulin concentrations [11].

Changes in Foetal Insulin Secretion with Intrauterine Growth Restriction

Human foetuses with severe intrauterine growth restriction (IUGR) have less pancreatic endocrine tissue and exhibit β-cell dysfunction [22, 27]. Such defects, if permanent, might limit β-cell function in later life and contribute to the increased incidence of non-insulin-dependent diabetes mellitus in individuals who were subject to growth restriction *in utero* [13, 14]. Similar observations have been made experimentally. For example, in the maternal hyperthermia-induced placental insufficiency model of IUGR in sheep, plasma insulin concentrations in the IUGR fetuses are 64 % lower at baseline and 77 % lower after glucose-stimulated insulin secretion (GSIS) [18]. Modelling of changes in plasma insulin concentration in response to glucose and arginine stimulation revealed deficits in the insulin secretion rate in the IUGR fetuses. When tested in vitro, pancreatic islets from the IUGR foetuses secreted insulin in response to increasing glucose and potassium chloride (KCl) concentrations, but the mass of insulin released per IUGR islet was lower than control islets from normally grown fetuses, due to an 82 % reduction in their insulin content [15]. A deficiency in islet glucose oxidation, but not total utilization rate, was found at maximal stimulatory glucose concentrations (11 mmol l–1). Interestingly, however, these same studies showed that insulin release as a fraction of total insulin content was greater in the IUGR islets. Thus pancreatic islets from placental insufficiency-induced IUGR foetuses have impaired β-cell stimulus–secretion coupling as a result of reduced glucose-stimulated glucose oxidation rates, insulin biosynthesis and insulin content, despite increased fractional rates of insulin release that results from a greater proportion of releasable insulin in the presence of lower insulin stores [16, 18].

Changes in Foetal Insulin Secretion with Hypoglycaemia

A common biochemical characteristic of fetuses with IUGR is relative hypoglycaemia; it is reasonable therefore to consider whether foetal pancreatic adaptations to hypoglycaemia alone, without other general nutrient deficiencies associated with IUGR (reduced amino acid and oxygen delivery, for example), would, by itself, limit the capacity of foetal pancreatic β-cells to produce and/or secrete insulin. Preliminary studies measured glucose-stimulated insulin secretion (GSIS) in normal control foetal sheep, foetal sheep made hypoglycaemic by maternal insulin infusion for 2 weeks in late gestation, and in a similar hypoglycaemic group of fetuses after a 5-day euglycaemic recovery period [16]. Hypoglycaemia significantly decreased plasma insulin concentrations in the hypoglycaemic (0.13}0.01 ng ml−1) and recovery foetuses (0.11}0.01 ng ml−1); insulin concentrations returned to euglycaemic control values (0.30}0.01 ng ml−1) in recovery fetuses (0.29}0.04 ng ml−1) during their euglycaemic recovery period. Mean steady state plasma insulin concentration during the GSIS study was reduced in hypoglycaemic fetuses (0.40}0.07 versus 0.92}0.10 ng ml−1 in controls); there was some recovery [19, 20].

Other Aspects of Glucose and Amino Acid Metabolism in IUGR

Up-regulation of mechanisms regulating glucose utilization. Many different models of IUGR [29] have shown that when the fetus is deprived of glucose, either selectively such as with maternal hypoglycaemia or generally as with placental insufficiency, foetal weight-specific glucose utilization rate is not very much different from normal rates [30]. These conditions both produce foetal hypoglycaemia, which increases the maternal–foetal glucose concentration gradient and thus the driving force for glucose transport across the placenta, thereby compensating in part for the reduction in glucose supply. For the fetus to accomplish this, there must be an increase in the foetal tissue capacity for glucose uptake and/or utilization, which could come about by increased concentrations and/or activity and/or plasma membrane localization of glucose transporters, increased insulin signal transduction and thereby effectiveness to promote Glut 4 (and perhaps Glut 1) translocation to the cell membrane, or mechanisms of insulin metabolism into oxidative and/or non-oxidative pathways. In both foetal sheep and foetal rats with IUGR, Glut 1 and Glut 4 concentrations in myocardium, adipose tissue and skeletal muscle do not decrease with sustained hypoglycaemia [8, 25, 26], perhaps a positive adaptation to maintain glucose utilization despite hypoglycaemia.

According to other preliminary data (M. A. Anderson, J. Friedman, W. W. Hay Jr, unpublished results, 2001) in placental insufficiency-induced IUGR, foetal sheep have shown increased levels of insulin receptor protein and decreased levels of the insulin signal transduction inhibitors, P85 protein subunit of phosphatidylinositol 3 kinase (PI3K), which negatively regulates the effect of insulin to promote Glut 4 translocation from inactive intracellular storage pools to active sites in the cell membrane where it enhances glucose uptake across the cell membrane, and glycogen synthase kinase (GSK), which negatively regulates glycogen synthase and the synthesis of glucose into glycogen [23, 24]. Therefore, foetal tissues in these chronically IUGR foetuses had adapted to their hypoglycaemic environment by developing mechanisms to promote glucose uptake and utilization, possibly via enhanced insulin action.

Other Changes in Foetal Metabolism in Response to Glucose (and Thus Insulin) Deficiency

A number of mechanisms have been noted, including glucose production from glycogen breakdown and the substitution of amino acids for glucose oxidation with acute glucose deprivation. With chronic glucose deprivation, however, foetal gluconeogenesis develops [21] and protein breakdown diminishes, allowing conservation of amino acids in protein structure while growth remains

limited by the decrease in amino acid transfer to the fetus by the placenta [28, 7]. If such adaptations are linked to the decreased insulin signal transduction protein concentrations found in skeletal muscle and liver in IUGR fetuses, it is possible that chronic nutrient deprivation leading to growth restriction might portend limited capacity for protein growth in later life.

Conclusion

The fetus has considerable capacity to adapt metabolically to acute and chronic changes in glucose supply by relatively common and understandable mechanisms. Current review dwells on the molecular and physiological changes in glucose utilization capacity which could be reversed by selective re-introduction of nutrient (glucose or amino acids, or both) and hormonal (insulin or IGF-1 or both) supplies, directly by infusion into the fetus or indirectly by infusion into the mother.

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Growth and Maturation of the Human Fetal Endocrine System Up to Twenty Four Weeks of Gestation

23

Subhankar Chowdhury

The fetus is nourished by a continuous supply of carbohydrates, amino acids, essential fatty acids, calcium, and oxygen in a warm environment, protected from outside influences by the fluid surrounding the fetus (amniotic fluid) which acts as a hydrostatic brace. However, at the time of birth, the fetus must have grown and developed, and its physiological systems must have sufficiently matured to survive a given level of parental care. In terms of fetal development, "maturation" refers to changes that facilitate survival following the transition from intra- to extrauterine existence. The human fetus must undergo endocrine maturation to generate hormones to support normal development and whose abnormal development/function can impact various systems with effects ranging from functional or structural defects to even death. A bouquet of transition factors and epigenetic events act hand in hand with autocrine, paracrine, and endocrine network of hormones and growth factors leading to the evolution of the fetal endocrine system with the hypothalamus-pituitary system providing the pivotal controlling template. Endocrine maturation commences early in gestation and continues till term and even thereafter to prepare the fetus for the external world. Most of the evidence available in this regard comes from animal studies with actual human data being available only in certain steps of this process. This chapter will look at the endocrine maturation of the human fetus up to 20 weeks of gestation and will also dwell upon its aberrations and subsequent implications.

Pituitary Gland

The anterior and intermediate lobes of the pituitary gland are derived from oral ectoderm, whereas the posterior pituitary is derived from neural ectoderm [1].

Anterior Pituitary

Anterior pituitary develops in four distinct stages beginning at around 4–6 weeks of gestation [2, 3].

Stage 1 (Pituitary placode). The anterior neural ridge is displaced ventrally to form the oral epithelium which forms the roof of the oral cavity. Onset of pituitary organogenesis coincides with a thickening (pituitary placode) in the roof of the oral ectoderm.

Stage 2 (Rudimentary Rathke's pouch). Invagination of the oral ectoderm forms a rudimentary pouch, and evagination of the

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ventral diencephalon forms the posterior pituitary. The pituitary placode makes contact with the floor of the ventral diencephalon. Apposition between the rudimentary Rathke's pouch and neural ectoderm of the diencephalon is critical to normal development of the anterior pituitary.

Stage 3 (Definitive Rathke's pouch). The rudimentary Rathke's pouch deepens and folds on itself until it closes and forms a definitive pouch (incomplete closure may persist as Rathke's cleft). The pituitary stalk is formed by evagination of the posterior part of the presumptive diencephalon.

Stage 4 (Adult gland). The Rathke's pouch is completely separated from the oral cavity. The final formation of a complicated secretory organ containing five different cell types occurs under the influence of several signaling and transcription factors over space and time. Remnants of pituitary tissue may persist in the nasopharyngeal midline and may rarely give rise to functional ectopic hormone-secreting tumors in the nasopharynx.

Critical neuroectodermal signals for initiating dorsal pituitary morphogenesis include infundibular bone morphogenetic protein 4 (BMP4), which is required for initial pouch invagination and also fibroblast growth factor 8 (FGF8), Wnt5 and Wnt4 [4, 5]. BMP4 and FGF8 are present only in the diencephalon which underlines the importance of apposition of the rudimentary Rathke's pouch and diencephalic neural ectoderm [6]. Subsequent ventral development is determined by expression of factors like BMP2 and sonic hedgehog protein (Shh). Early cell differentiation requires intracellular Rpx (HESX1) and Ptx (PITX) expression. Rathke's pouch expresses transcription factors like Lhx3, Lhx4, and IsI-1 which are required for progenitor cell survival and proliferation. Ptx1 is expressed in the oral ectoderm, and subsequently in all pituitary cell types, particularly those arising ventrally while Ptx2 mutation causes Rieger's syndrome, characterized by defective eye, tooth, umbilical cord, and pituitary development. Ptx is an universal pituitary regulator and activates transcription of αGSU, POMC, LHβ (Ptx1), and GH (Ptx2). Lhx3 determines GH-, PRL-, and TSH-cell differentiation. PROP1 acts as a prerequisite for POU1F1, which activates GH, PRL, TSH, and growth hormone– releasing hormone (GHRH) receptor transcription. TSH and gonadotropin-expressing cells share αGSU expression under developmental control of GATA2 and FOXL2. Pituitary cell type diversification is mediated by binary Wnt/β-catenin signaling which causes induction of PROP1 and suppression of HESX1. PROP1 expression is particularly important as it determines subsequent development of POU1F1-dependent (GH, PRL, TSH, and GHRH receptor) and gonadotroph cell lineages [7]. Signal-dependent coactivating factors also cooperate with POU1F1 to determine specific hormone expression (Estrogen induces PRL while TEF induces TSH expression). Steroidogenic factor (SF1) and DAX1 (encoded by NROB1) determine subsequent gonadotroph development [8, 9]. Corticotroph cell commitment occurs earliest during fetal development and requires TPIT protein (encoded by TBX19) for POMC expression. Table 23.1 gives an outline of the syndromes arising from mutation in these transcription factors [6].

Corticotroph cells are morphologically identifiable at 6 weeks and immunoreactive ACTH is detectable by 7 weeks. Somatotroph cells are subsequently evident with abundant immunoreactive cytoplasmic GH expression at 8 weeks. Nine- to sixteen-week-old human fetal pituitary cells in culture show a predominant response to GHRH and a limited effect of somatostatin, suggesting that the inhibitory action of somatostatin develops later in gestation [10]. At 12 weeks, differentiated thyrotrophs and gonadotrophs express TSH, LH and FSH, respectively. Immunoreactive PRL is detectable in mixed mammosomatotrophs along with GH in the early half of gestation and pituitary PRL content increases progressively from 12 to 15 weeks. PRL receptors are present in most fetal tissues during first trimester and are implicated in fetal

Table 23.1 Genetic mutations and alterations in pituitary development

Gene	Phenotype
HESX1	Variable: septo-optic dysplasia, combined pituitary hormone deficiency, isolated growth hormone deficiency (IGHD) with ectopic posterior pituitary (EPP)
	Anterior pituitary hypoplastic or absent
	Posterior pituitary ectopic or eutopic
	Frequency of mutations: <1 %
OTX2	Anophthalmia, anterior pituitary hypoplasia (APH), EPP, absent infundibulum
	Frequency of mutations: 2–3 % of anophthalmia/microphthalmia cases
SOX2	Hypogonadotropic hypogonadism; APH, abnormal hippocampi, bilateral anophthalmia/microphthalmia, abnormal corpus callosum, learning difficulties, esophageal atresia, sensorineural hearing loss, hypothalamic hamartoma
	Frequency of mutations: 3.4 %
SOX3	IGHD and mental retardation, hypopituitarism; APH, infundibular hypoplasia, EPP, midline abnormalities
	Frequency of mutations: 6 % (duplications), 1.5 % (mutations)
GLI2	Holoprosencephaly, hypopituitarism, craniofacial abnormalities, polydactyly, single nares, single central incisor, partial agenesis of corpus callosum
	Frequency of mutations: 1.5 %
LHX3	GH, TSH, gonadotropin deficiency with pituitary hypoplasia
	ACTH insufficiency variable
	Short, rigid cervical spine
	Variable sensorineural hearing loss
	Frequency of mutations: 1.3 %
LHX4	GH, TSH, cortisol deficiency, persistent craniopharyngeal canal and abnormal cerebellar tonsils; APH, ectopic/eutopic posterior pituitary, absent infundibulum
	Frequency of mutations: 1.2 %
PROP1	GH, TSH, PRL, and gonadotropin deficiency
	Evolving ACTH deficiency
	Enlarged pituitary with later involution
	Frequency of mutations: 1.1 % sporadic cases, 29.5 % familial cases
POU1F1	Variable anterior pituitary hypoplasia with GH, TSH, and PRL deficiencies
	Frequency of mutations: 3.8 % sporadic cases, 18 % familial cases

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growth, skeletal maturation and adipose tissue maturation [11, 12].

The hypothalamic cell condensations which are the precursors of the hypothalamic nuclei, and the interconnecting fiber tracts are demonstrable by 15–18 weeks of gestation [3]. Concentrations of dopamine, TRH, GnRH, and somatostatin are significant in hypothalamic tissue by 10–14 weeks of gestation. Capillaries develop within the proliferating anterior pituitary mesenchymal tissue around Rathke's pouch by 8 weeks of gestation, and intact hypothalamic-pituitary portal vessels are present by 12–17 weeks but the maturation process extends almost till end gestation.

Intermediate Lobe of the Pituitary

The intermediate lobe is prominent in early gestation and is virtually absent in the adult human pituitary [13]. The major secretory products of the intermediate lobe are α-MSH and β-endorphin but the role of these intermediate lobe peptides in the fetus remain obscure [14].

Posterior Pituitary

The fetal neurohypophysis is well developed by 10–12 weeks of gestation and contains both arginine vasopressin (AVP) and oxytocin (OT) [15]. In addition, arginine vasotocin (AVT) is present in the fetal pituitary and pineal gland but its actual role is unknown [16]. AVP functions in the fetus mainly as a stress-responsive hormone responding to stress like hypoxia while AVP response to osmolar stimuli and its antidiuretic effects are prominent during the last trimester of gestation [15].

Adrenal System

Understanding adrenal embryogenesis is mostly derived from studies in transgenic mice and humans with various forms of adrenal hypoplasia. The primordia of the adrenal glands can be recognized just above the bilaterally developing mesonephros by 3–4 weeks of gestation [17, 18]. The adrenal cortex is derived from a thickening of the intermediate mesoderm at 4–5 weeks of gestation while the adrenal medulla originates from the ectoderm. This region is known as the gonadal ridge and gives rise to the steroidogenic cells of both the adrenal gland and the gonads. Early differentiation of the adrenogonadal primordium from the urogenital ridge requires signaling cascades and transcription factors GLI3, SALL1, FOXD2, WT1, PBX1, WNT4, and the regulator of telomerase activity, ACD. Separation of the adrenogonadal primordium and formation of the adrenal primordium depend on the actions of transcription factors SF1, DAX1, WNT4, and CITED2. Among these, SF1 and DAX1 play a particularly important role in early adrenal development [19]. DAX1 probably acts as a repressor of SF1 transcription and regulates adrenal progenitor cell development and maturation. Adrenal tissue precursors migrate retroperitoneally to the upper pole of the mesonephros and are infiltrated at 7–8 weeks of gestation by sympathetic cells derived from the neural crest that subsequently form the adrenal medulla. The adrenocortical primordium develops at approximately 8 weeks of gestation and can differentiates into two distinct layers, the inner fetal zone (FZ) and the outer definitive zone (DZ) which depends on the temporal expression of transcription factors including Pref-1/ZOG, inner zone antigen, and SF1 [20]. Encapsulation of the adrenal gland occurs after 8 weeks of gestation and results in the formation of a distinct organ just above the developing kidney.

The fetal adrenal has three functional zones – an outer definitive zone capable of producing both glucocorticoids and mineralocorticoids, a transitional zone with enzymes for cortisol production, and a much larger inner fetal zone capable of producing significant amounts of C19 androgens such as dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS), which are then converted to estrogens by the placenta. The large eosinophilic cells of the fetal zone are well differentiated by 9–12 weeks of gestation and are capable of active steroidogenesis. Quantitative differences in the relative activities of the five steroidogenic apoenzymes are found between cells derived from the fetal versus the definitive zones due to regulated steroidogenic gene transcription. The fetal zone has relatively high steroid sulfotransferase activity which together with low 3β-HSD results in the predominant production of DHEA, DHEAS, pregnenolone sulfate, several Δ5 3β hydroxysteroids, while only limited amounts of Δ5 3-ketosteroids, including cortisol and aldosterone are produced by the fetal adrenals [17, 18]. This programming is designed to provide DHEA substrate for placental estrone and estradiol production. The fetal zone contains more LDL binding sites and manifests a greater rate of de novo cholesterol synthesis than does the definitive zone, in keeping with its greater steroidogenic activity. Glucocorticoids are synthesized in the first trimester due to transient expression of type 2 3β-hydroxysteroid dehydrogenase (HSD3B2) using pregnenolone as the precursor instead of denovo cholesterol synthesis [21]. Fetal pituitary ACTH stimulates steroid production by activating StAR and increasing delivery of substrate cholesterol to P450scc and is the major stimulus to adrenal function as evidenced by the reduction

of placental lactogen production by 90 % and the involution of the adrenal gland after 15 weeks in the anencephalic fetus [17, 21]. Angiotensin II inhibits 3β-HSD activity and promotes DHEA production in the fetal zone in early gestation.

Glucocorticoid receptors (GR) are present at midgestation in most tissues, including placenta, lung, brain, liver, and gut and play an important role in fetal development. Mice lacking GR function manifest enlarged and disorganized adrenal cortices, adrenal medullary atrophy, lung hypoplasia, and defective gluconeogenesis [22]. Fetal cortisol is converted to inactive cortisone through 11ßHSD2 in fetal tissues and this metabolism protects the anabolic milieu of the fetus, as cortisol can retard both placental and fetal growth [23]. Mineralocorticoid receptors (MR) are present from 12 to 16 weeks in fetal kidney, skin, hair follicles, trachea and bronchioles, esophagus, colon, small intestine, and pancreatic exocrine ducts but their role remains unclear with aldosterone secretion remaining low in midgestation and being unresponsive to secretagogues [24].

Adrenal insufficiency may be secondary to ACTH deficiency or may be due to primary adrenal failure. ACTH deficiency may be isolated (TPIT mutation) or a part of multiple pituitary hormone deficiency (discussed earlier in Table 23.1). Recessive mutations in the T-box factor *TPIT* (TBX19) have been identified in patients with severe, early-onset isolated ACTH deficiency with profound hypoglycemia, prolonged jaundice, and sudden neonatal death [25]. TPIT is required for the specification, maturation, and maintenance of both precorticotrope and premelanotrope populations and for the suppression of gonadotrophs. It is also required to activate the expression of POMC in conjunction with the transcription factor PTX1. SF1 gene mutation causes adrenal and gonadal agenesis, gonadotropin deficiency, and absence of the hypothalamic ventromedial nucleus [26]. Mutations in SF1 have been associated with gonadal dysgenesis in 46XY individuals without any adrenal involvement and have also been associated with primary ovarian failure.

Inactivating DAX1 gene mutations are associated with adrenal hypoplasia and gonadotropin deficiency [26]. In humans, inactivating *StAR* mutations cause adrenal hypoplasia and adrenal hormone insufficiency, and normal to varying degree of ambiguous genitalia in XY males [27]. Various forms of congenital adrenal hyperplasia may be associated with variable adrenal failure (e.g., mutations in CYP11A1, StAR, HSD3B2I, CYP17, CYP21, CYP11B1) with varying degrees of genital ambiguity [28, 29].

Autonomic Nervous System

The paired adrenal glands are well developed by 10–12 weeks of gestation. There is progressive growth and maturation of the adrenal medulla with increasing gestational age and histological maturation is complete only at 1 year of postnatal life [6]. In the adrenal glands, the progenitor cells derived from common neuroectodermal stem cells differentiate into neuroendocrine cells, expressing tyrosine hydroxylase and dopamine β-hydroxylase in response to a series of transcription factors including PHOX2B, MASH1, PHOX2A, and dHAND [30, 31]. The *PHOX2B* gene plays a central role in the development of most relays of the autonomic nervous system and its mutation has been associated with the congenital hypoventilation syndrome, Hirschsprung's disease, and a predisposition to neuroblastoma [32]. Catecholamines are present in the chromaffin tissue by 10–15 weeks of gestation and the adrenal medulla can respond directly to asphyxia, long before splanchnic innervation develops, by secreting norepinephrine. Catecholamines are critical for fetal cardiovascular function and fetal survival and its absence leads to midgestation fetal death in the majority [33]. Catecholamines are also the major stress hormone in the fetus and is discharged in large amounts into the circulation in response to fetal hypoxia [34]. Also, the defense against fetal hypoxia involves action of catecholamine on the cardiac α-receptors which predominate early in gestation and gradually decline in number as β-adrenergic receptors increase.

Thyroid

The pharyngeal floor thickens in the midline (median anlage) and acts as the precursor of the T4-producing follicular cells while paired caudal extensions of the fourth pharyngobranchial pouches (lateral anlagen) give rise to the parafollicular calcitonin-secreting cells (C cells) [35, 36]. These structures are identifiable by 16–17 days of gestation, and by 24 days the median anlage develops a thin, flask-like diverticulum extending from the buccal floor to the fourth branchial arch. The primitive structure connecting the primordium to the pharyngeal floor elongates into the thyroglossal duct. At 24–32 days, this median anlage is a bilobed structure, and by 50 days of gestation, the median and lateral anlagen fuse with each other. Dissolution and fragmentation of the thyroglossal duct usually occurs by 37–50 days and a small dimple is left at the point of origin of the thyroglossal duct at the junction of the middle and posterior thirds of the tongue which is known as the foramen caecum. Cells of the lower portion of the thyroglossal duct differentiate into thyroid tissue, forming the pyramidal lobe of the gland [37]. Meanwhile, the thyroid gland migrates caudally from the pharyngeal floor, through the anterior midline of the neck, to its definitive location in the anterior neck. At 51 days, the gland exhibits its definitive external form, with an isthmus connecting the two lateral lobes, and it reaches its final position below the thyroid cartilage by the seventh week of embryonic life. An ectopic thyroid and persistent thyroglossal duct or cyst may occur as a consequence of abnormalities of thyroid descent. Rarely, thyroid tissue may develop from remnants of the thyroglossal duct near the base ofthe tongue forming a lingual thyroid tissue which may be the sole functioning thyroid present [37]. This has clinical importance as its inadvertent removal may lead to iatrogenic hypothyroidism.

A Tbx1-Fgf8 pathway in the pharyngeal mesoderm plays a key role in early thyroid migration and development. Several developmental genes are involved in thyroid and parathyroid gland embryogenesis. These include the genes for thyroid transcription factors HEX, TTF1 (Titf1/Nkx2.1), FOXE1 (Titf2/Foxe1), NKX2-5, and PAX8 [38–40]. HEX gene mutation is associated with thyroid agenesis or severe hypoplasia. TTF2 mutation results in thyroid dysgenesis and cleft palate while TTF1 defect leads to pulmonary hypoplasia, parafollicular C-cell aplasia and thyroid agenesis. Inactivating Pax8 mutations lead to thyroid hypoplasia and renal anomalies. TTF1/NKX2.1 and PAX8 also play a role in the survival of thyroid cell precursors and regulation of thyroid specific gene expression, while FOXE1/TTF2 is essential for cellular migration. The SHH gene may have an important role in later stages of thyroidogenesis in the symmetric bilobation of the gland and it also suppresses the ectopic expression of thyroid follicular cells. However, mutations in these genes account for fewer than 10 % of patients with familial thyroid dysgenesis and congenital hypothyroidism and most cases occur sporadically and are of unknown aetiology.

Thyroglobulin (Tg) starts forming in the future follicular cells from as early as the 29th day of gestation, while the capacities to concentrate iodide and synthesize thyroxine (T4) are delayed until about the 11th week [41]. Radioactive iodine given inadvertently to the mother before this period is not actually harmful while later administration may ablate the fetal thyroid. Early growth and development of the thyroid is not dependent on TSH as pituitary TSH secretion is not apparent until the 14th week. Thyroxine-binding globulin (TBG), the major thyroid hormone–binding protein in plasma, is detectable by the tenth gestational week and increases in concentration progressively till term. The terminal differentiation of thyroid follicular cells as evidenced by expression of the genes encoding the TSH receptor (TSHR), the sodium-iodide symporter (NIS), thyroglobulin (Tg), and thyroperoxidase (TPO) and the formation of follicles – occurs in the normal embryo only after migration is complete [42]. Genes encoding Tg, TPO, and pendrin are expressed as early as 7 weeks' gestational age.

NIS expression appears last from 11th gestational week onwards suggesting a key role of NIS in the onset of thyroid function while Tg is detectable in unpolarized thyrocytes before follicle formation [43].

Classic thyroid hormone actions are mediated via functional thyroid hormone nuclear receptors- TRα1, TRα2, TRβ1, and TRβ2. TRα1 and TRβ1 isoforms and receptor binding are present by 8–10 weeks of gestation in the fetal brain with TRα transcription and receptor occupancy increasing eight- to tenfold by 16–18 weeks. Liver, heart, and lung receptor binding can be identified by 13–18 weeks of gestation [36].

In early gestation, placental transfer of T4 is the only source of thyroid hormone and is critical for fetal neurodevelopment playing an important role in neurogenesis and neural cell migration, neuronal differentiation, dendritic and axonal growth, synaptogenesis, myelination, and neurotransmitter enzyme synthesis [44]. Most of the thyroid hormone in the fetal compartment is inactivated to sulfated and deiodinated analogues until the perinatal period to maintain a low T3 metabolic state, facilitating fetal growth and programmed tissue maturation [45]. In congenital hypothyroidism, cord T4 concentrations are 25–50 % of normal due to an increased net transplacental flux of maternal thyroid hormone. The transplacental passage of thyroid hormone along with alteration in brain deiodinase activity plays a crucial role in minimizing the adverse effects of fetal hypothyroidism and helps explain the near-normal outcome of hypothyroid fetuses (with adequate postnatal levothyroxine supplementation). On the other hand, in the presence of both maternal and fetal hypothyroidism (due to potent TSH receptor–blocking antibodies, maternal and fetal POU1F1 deficiency, and severe iodine deficiency), there is severe neurocognitive impairment despite early and adequate commencement of thyroid replacement. Significantly, the presence of maternal hypothyroxinemia or inadequately controlled hypothyroidism results in significant and irreversible neurocognitive deficit in the offspring [46, 47].

Parathyroid Gland

Inferior and Superior parathyroid glands develop from the third and fourth pharyngeal pouches respectively in a co-ordinated fashion with thyroid gland formation [35]. The parathyroid anlage of the third pouch is carried caudally with the migrating thyroid anlage and ends up at the lower poles of the thyroid lobes as the inferior parathyroid glands. The fourth pouch encounters the thyroid anlage later and comes to rest at the upper poles of the thyroid lobes as the superior parathyroid glands. HOX15 plays a pivotal role in the gene cascade programming normal thyroid-parathyroid gland development and its silencing results in parathyroid gland aplasia. Genes like SOX3, GCM2, GATA3, CRKL, and TBX1 are also involved in the development of the parathyroid gland [47, 48]. CRKL and TBX1 mutations are associated with the DiGeorge syndrome and GATA3 mutation gives rise to a DiGeorge-like syndrome while GCM2 mutation leads to isolated hypoparathyroidism. Transplacental calcium transfer maintains high fetal calcium levels and occurs across the syncytiotrophoblast, which contains a calcium-binding protein that buffers intracellular calcium ions as they are transported across the syncytial cell to the basement membrane [49]. A placental ATP-dependent calcium pump transports the calcium to the fetal circulation and is stimulated by a midmolecule portion of PTHrP (via an unidentified receptor) secreted by the fetal parathyroid gland and by the placenta, via a paracrine effect [50]. Absence of fetal parathyroids due to any cause results in a low fetal plasma calcium concentration and a loss of the placental calcium gradient. PTH and PTHrP, through the PTH/PTHrP receptor, probably modulates fetal skeletal calcium flux, calcium excretion through the fetal kidney, stimulates fetal renal 1,25(OH)2D production and reabsorption of calcium from amniotic fluid. PTHrP has a major role in fetal bone development and metabolism as well as fetal calcium homeostasis and its absence results in neonatal death due to severe and widespread bony defects. Chronic stimulation by fetal hypercalcemia results in high blood levels of calcitonin which inhibits bone resorption and promotes bone mineral anabolism [51].

Gonadal System

Two anlagens give rise to the gonads – the primordial germ cells of the yolk sac wall and the somatic, stromal cells that migrate from the primitive mesonephros [52, 53]. Germ cells begin their migration from the yolk sac by 4–5 weeks of gestation when the gonadal ridge appears as a derivative of the mesonephros. The germ cells get included in the developing gonadal ridge during the sixth week. The primitive gonad is composed of a surface epithelium, primitive gonadal cords continuous with the epithelium, and a dense cellular mass referred to as the gonadal blastema. Primordial cells that fail to migrate normally may account for the location of extragonadal germ cell cancers in men. The fetal testis and ovary are indistinguishable until 6 weeks when the testicular cords made up of Sertoli cells appear under the influence of the Y chromosome. The SRY (sex-determining region of the Y chromosome) gene on the pseudoautosomal region of the Y chromosome encodes a transcription factor that increases the expression of SRY-box 9 (SOX9), which directs formation of Sertoli cells and testis differentiation [54]. The primitive cords lose their connections with the epithelium, primitive Sertoli cells and spermatogonia become visible within the cords, and the epithelium differentiates to form the tunica albuginea. SRY gene expression is activated by steroidogenic factor 1 (SF1) and the binding protein GATA4 [55]. SF1 also results in SRY-independent SOX9 expression, AMH gene expression and gonadotropin production. SOX9 drives expression of other genes like fibroblast growth factor 9 (FGF9) and AMH that are necessary for differentiation of testis and genes like WNT4 and NROB1 which repress ovary formation. WT1 is expressed in the genital ridge and its expression mediates the transition of mesenchyme to epithelium. In XX individuals, SOX9 is repressed by factors like β-catenin leading to the development of the follicular cells and ovaries. Leydig cells begin to form at 8 weeks of gestation from the undifferentiated interstitium and make up about 50 % of the cell mass at 14 weeks [56]. The differentiation, proliferation, and organization of the gonads occur initially under the influence of maternal human chorionic gonadotropin (hCG) and later LH and FSH from the fetal pituitary gland. Testosterone production from fetal Leydig cells increases progressively between 10 and 20 weeks and induces development of the male internal genitalia while conversion of testosterone to 5α-dihydrotestosterone (5α-DHT) in the urogenital tract leads to the formation of the prostate and male external genitalia [57]. Androgen receptors appear in the mesenchyme of urogenital structures at 8 weeks of gestation, followed by appearance of the receptors in the epithelium during development at 9–12 weeks. Male phenotypic development is complete by around 15 weeks of gestation. The developing testis is attached to the diaphragm by the craniosuspensory ligament and anchored to the inguinal region by a caudal ligament known as the gubernaculum. The initial transabdominal phase of testicular descent occurs between 10 and 23 weeks of gestation and depends on two processes: (1) testosterone induced regression of the craniosuspensory ligament (2) thickening of the gubernaculum, which is controlled by INSL3 produced by Leydig cells and its cognate receptor, relaxin family peptide receptor 2 (RXFP2, also known as leucine-rich repeat–containing G protein–coupled receptor 8 [LGR8] or G protein–coupled receptor affecting testis descent [GREAT]) [58]. AMH secretion from the fetal Sertoli cells causes regression of the Müllerian ducts preventing the formation of uterus and fallopian tubes while female genitalia develop in the absence of testosterone. AMH gene expression is activated by the SRY and SF1 genes and also has autocrine and paracrine effects on fetal testicular steroidogenic function. AMH mutation results in persistent Mullerian duct syndrome in the XY fetus [59].

Loss-of function mutations of SRY or SOX9 produce XY sex reversal, whereas gain-of-function mutations or duplications produce XX sex reversal [53]. SOX9 mutations may also cause camptomelic dysplasia. SF1 mutations may lead to 46XY sex reversal with gonadal dysgenesis, with or without adrenal failure and may also result in vanishing testes syndrome. WT1 mutations produce several syndromes associated

with abnormal testicular embryogenesis (WAGR syndrome [Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation], Denys-Drash syndrome, and Frasier syndrome) and renal abnormalities such as Wilms' tumor or glomerulosclerosis. WNT4 gain-of-function mutations result in XX sex reversal, whereas NROB1 duplication is associated with XY sex reversal.

In females, in the absence of SRY, the gonadal blastema differentiates into interstitium and medullary cords containing the primitive germ cells (oogonia). By 11–12 weeks of gestation, clusters of dividing oogonia are surrounded by cord cells within the cortex while the medulla at this time consists largely of connective tissue [60]. At 12 weeks of gestation, primitive granulosa cells begin to replicate and many of the large oogonia in the deepest layers of the cortex enter their first meiotic division. Primordial follicles are observed initially at about 18 weeks and the number increases rapidly thereafter although the number of oocytes progressively declines (from a peak of three to six million at 5 months' gestation) [61]. Interstitial cells capable of steroid production are present after 12 weeks although steroids are practically not produced by the developing ovary [21]. RSPO1 mutation may cause 46XX sex reversal as it belongs to the R-spondin family of proteins that is implicated in Wnt/β-catenin signaling, and the gene encoding the protein may determine ovary formation [62]. Estrogen receptors have been characterized in the 16- to 23-week human fetus. The ERβ message is commonly present, particularly in testis, ovary, spleen, thymus, adrenal, brain, kidney, and skin. The ERα message is prominent in the uterus, with relatively low levels in most other tissues. However, the clinical significance of ERs remains uncertain as ER knockout laboratory animals are normal at birth [63].

Endocrine Pancreas

Pancreatic budding occurs from the gut tube and differentiation of exocrine and endocrine cell lineages occur from endodermal tissue under the influence of a number of genes and transcription factors [6, 64]. SOX9 and HNF3B are required for early foregut formation and pancreas specification while PDX1, HLXB9, and ISL1 play a role early in pancreatic development. HES1 and neurogenin 3 (NGN3) take part in development of the islets of Langerhans. Pancreatic agenesis may result from PDX1 mutation causing neonatal diabetes mellitus. The human fetal pancreas is identifiable by 4 weeks of gestation, and alpha and beta cells can be recognized by 8–9 weeks. Insulin, glucagon, somatostatin, and pancreatic polypeptide are measurable by 8–10 weeks of gestation [65]. Alpha cells outnumber the beta cells in the early fetal pancreas and reach peak at midgestation, while beta cells increase in the latter half of gestation and by 20 weeks, endocrine cells are dispersed throughout the exocrine tissues. The fetal beta cell begins to function from around the 14th week but to a very limited extent such that the secretion of insulin into the bloodstream by the fetal pancreas is low. Development of the pancreas is independent of the pituitary as the endocrine pancreas develops normally in anencephalic fetuses; however, beta cell hyperplasia and hypertrophy to hyperglycemia exposure is lacking which may be due to deficiency of GH-IGF-1, which plays a permissive role otherwise [66]. Pancreatic glucagon content is high in midgestation but its secretion is blunted in the fetus. The blunted capacity for fetal insulin and glucagon secretion may be on account of the relatively stable fetal serum glucose concentrations maintained by transplacental maternal glucose transfer, lack of enteric incretin signal to the pancreas from feeding and deficient cAMP in the fetal pancreatic islet due to deficient production or rapid destruction. In early gestation, hepatic metabolism and substrate utilization are insulin-independent and modulated in an autoregulatory fashion by glucose, while glycogen storage in the fetus is modulated by fetal glucocorticoids and probably by placental lactogen [67].

The above knowledge and ongoing research has set the stage for fetal endocrine disease diagnosis, therapy for fetal endocrine and metabolic disorders and diagnosis and management of neonatal endocrine dysfunction. Moreover, manage-

ment strategies for premature infants and infants and children with fetal growth restriction can be better devised with an understanding of endocrine maturation. We are on the doorstep of an era of direct management of the intrauterine environment both medically and surgically for which an elaborate understanding of fetal development is of prime importance. Intrauterine diagnosis and treatment of fetal adrenal and thyroid disorders are already in vogue and increasing availability of synthetic hormones and growth factor agonists and antagonists facilitate direct fetal endocrine therapy [68, 69]. In early gestation, the fetus is a favorable recipient of stem cells and this technology may be applicable to therapy for selected endocrine and metabolic diseases [70]. Cutting edge research has increased our experience with fetal gene therapy in animals and in the near future, similar therapy may be a reality in humans too.

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Part VI

Fetal Immune Development: Up to Second Trimester

Development of Human Immune System in First and Second Trimester

24

Swapna Chaudhuri

Introduction

The immune paradox lies in the fact that the semi-allogenic embryo (or allogenic embryo in surrogate mothers) is not immunologically rejected [95]. A pregnancy may be spontaneously aborted in 10–20 % of cases without any apparent reason [2, 60]. There are multiple sets of immunological needs for the fetus and newborn, like protection against infection and bypassing harmful inflammatory immune responses that can lead to pre-term delivery, and also the transition from a sterile intra-uterine environment to external world full of unknown antigens. These immunological needs shape neonatal innate immune system dampening the production of pro-inflammatory cytokines rendering newborns at risk of infection and impairing responses to many vaccines [63]. Ontogeny of human fetal immune system in mid-term fetus is derived from a completely different set of stem cells than the adult immune system. Fetal immune system can tolerate antigens (including its mother and its own organs) better than to eliminate antigens from its environment. Midterm fetal immune system has about three times more regulatory T cells than newborns or adults, which render tolerance [75]. Treg blocked fetal T-cell responses to maternal cells.

Trophoblast-derived thymic stromal lymphopoietin (TSLP) instructs decidual CD11c+ dendritic cells (dDCs) with increased costimulatory molecules; MHC class II; and Th2/3-type, but not Th1-type, cytokines. TSLP-activated dDCs cause proliferation and differentiation of decidual CD4+ CD252 T cells into CD4+ CD25+ FOXP3+ regulatory T cells (Tregs) through TGF-b1 [26]. Decidual CD4+ CD25+ FOXP3+ Tregs promote invasiveness and HLA-G expression of trophoblasts, resulting in preferential production of Th2 cytokines and reduced cytotoxicity in decidual CD56brightCD16 NK cells [26].

It is of primary need to strengthen all the factors to protect the mother and the neonate since, pregnancy represents the most important period for the conservation of the species. To protect the mother against the environment and preventing damage to the fetus the immune system plays an important role. The maternal immune system during pregnancy is characterized by a reinforced network of recognition, communication, trafficking and repair; to maintain the well-being of the mother and the fetus it is able to raise the alarm, if necessary. On the other side, the fetus provides a developing active immune system that modifies the way the mother responds to the environment. Therefore, it is appropriate to refer to pregnancy as a unique immune condition that is modulated,

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but not suppressed. This unique behaviour explains why pregnant women respond differently to the presence of microorganisms or its products [76].

There are three threats to the embryo: (i) lysis or immobilization by anti-embryonic antibodies, (ii) attack by non-MHC-restricted cells, and (iii) ongoing maternal immune response directed towards the implantation site. Acceptance of the fetal "allograft" by the maternal immune system is one of the mysteries of the immune system. The active expression of T-cell immunity against HLA antigens expressed on fetal tissues, results in placental detachment and fetal loss. When reactivity is less intense, it may result in pre-eclampsia and premature delivery. In this regard T-cell responses are Th1-polarized and are dominated by IFN-γ, which is highly toxic to the placenta [114].

Absence of MHC antigens of human preimplantation embryo prevents direct attacks by MHC-restricted T cells. Maternal APC presenting paternal or embryo-derived peptides triggers CD4+ and CD8+ MHC effector T cells yielding vulnerability to the human embryo [17]. During the first trimester, NK cells, dendritic cells and macrophages infiltrate the decidua and accumulate around the invading trophoblast cells [4, 97]. uNK cells are critical for trophoblast invasion in the uterus and uDC are necessary for decidual formation and may affect the angiogenic response by inhibiting blood vessel maturation [7].

A series of overlapping control mechanisms selectively downregulate Th1 immunity at the fetomaternal interface and within the fetal microenvironment itself operate at the level of the placenta. Expression of FasL on fetal cells interact with Fas on activated T cells as a potential means of elimination of T cells by apoptosis [39, 40]. CD95 receptor mediates cell death signal for apoptosis. CD95 ligand has a crucial role in the homeostasis of haematopoietic cell populations in adults. Compared to adult peripheral blood lymphocytes the low level of CD95 ligand expression of cord blood mononuclear cells enjoy some immune privilege [25]. Local production of T-cell suppressive tryptophan metabolites via indoleamine 2,3-dioxygenase, which is expressed in syncytiotrophoblasts and macrophages [79]. Immunosuppressive molecules in the placenta, such as progesterone [88, 104, 105], prostaglandin E2 [1, 44] and early pregnancy factor [78] helps to prevent immunological rejection of an embryo. Moreover, Th2-trophic and/or Th1-suppressive factors are produced by the placenta in high levels including IL-4 and IL-10 [93]. Progesterone ensures that any environmental antigens/allergens that pass via the maternal circulation across to the developing fetus will elicit fetal immune system Th2 cytokines [89]. Trophoblasts express nonclassical MHC molecules HLA-G, HLA-C, HLA-E, and HLA-F [109]. HLA-G binds to the killing inhibitory receptor on NK cells and protects trophoblasts from NK cell–mediated attack. HLA-G may also protect the fetus from an allogeneic T cell response.

The commensal microflora of the gastrointestinal tract imparts signals from the microbial environment to stimulate postnatal maturation of the immune function in mammals. Infections, in the gastrointestinal and respiratory tracts, may also contribute to this process [46]. The principal focus of this late-stage maturation process is upregulation of Th1 functions, which, are dampened during fetal life. Absence of adequate microbial stimulation during infancy results in preponderance of Th2 phenotype, resulting in blunted expression of Th1 immunity at peripheral challenge sites [49], at mucosal surfaces a failure of the immune deviation mechanisms that normally regulate induction of Th2 responses [103], and excessive class switching of immature B cells toward IgE commitment [27]. The molecular signalling between the microbial environment and the immune system through the TOLL receptors [51, 116], as well as the high-affinity receptor for bacterial lipopolysaccharide (CD14), are found to be central in the process.

Development of the Fetal Immune System

At about gestational 6 weeks thymus arises from the third branchial arch and cortex arises from ectodermal layer and the medulla arises from the

endoderm. Over the next 2–3 weeks lymphoid cells migrate, initially from the yolk sac and fetal liver, and then from the bone marrow to colonize the fetal thymus [20, 46].

The initial stage of human fetal haemopoiesis occurs in the yolk sac mesoderm and the extra-embryonic mesenchymal tissue. At 3–4 weeks of gestation pluripotent erythroid and granulomacrophage progenitors appear in the yolk sac of human embryos. At 5–6 weeks of gestation these primitive cells can then be detected in the circulation, and from 4 weeks of gestation they migrate to the liver which becomes the major site of haemopoiesis. Liver size increases significantly with rise in the number of nucleated cells from the 5–10 weeks. Although these early progenitors keeps on proliferating very little differentiation occurs although a discrete granulocyte/macrophage population emerges at this time. The thymus and spleen are seeded from the liver and stem cells are detectable in the bone marrow at 11–12 weeks of gestation [73]. Hepatic haematopoiesis declines in the third trimester of pregnancy but ceases soon after birth.

Fetal blood collected by fetoscopy at 12–19 weeks of gestation when cultured yields high levels of both erythroid and granulocytic/monocytic progenitor cells – monocytes comprising 42–68 %, neutrophils 27–41 %, and eosinophils 5–30 % [65]. Although the number of granulocyte progenitors in the circulation is high at this time, granulocytes are not formed in significant amount in the foetuses until after birth. Neutrophils appear last in the blood during fetal life [32].

Developmental Origin of Hematopoietic Stem Cells (HSCs)

The first wave of hematopoiesis occurs in the yolk sac in the first month [87] and yields initially macrophages and mature red blood cells that remain nucleated before the circulation of blood begins [85]. Mature and more primitive hematopoietic cell including the first lymphoid cells generate subsequently [11, 50]. Between weeks 7 and 19 of gestation [12] a significant number of committed and multi-potent CD34+ progenitors with a capacity for expansion circulate in the fetal blood. Later in midgestation, cells that display the self-sustaining properties of HSCs arise predominantly from hemogenic cells with endothelial features lining both dorsal aorta and blood vessels in the placenta [28]. These HSCs then translocate to the fetal liver and spleen with rapid amplification. Just before birth they migrate again to developing niches in the bone marrow where HSCs remain concentrated throughout adult life, although some may also recirculate [77]. The HSCs migration during embryogenesis in the bone marrow microenvironment is controlled by adhesion receptors including CD34, CD44, selectins, and integrins [48], α4β1 (VLA-4) and its ligand VCAM-1 are involved in the homing of hematopoietic progenitors to spleen and bone marrow [86]. In the developing fetal liver, the lymphoid-deficient α-HSCs constitute only less than 5 % of all HSCs and less than 10 % of the HSCs with durable self-renewal ability. However, these are much more prevalent (~3× more) amongst the initial HSC population that can be detected in fetal bone marrow (BM) just before birth [5].

In normal pregnancy, the rate of fetal mononuclear-cell division rate decreases with gestational age from 1.8 % at 18 weeks to 1 % at 40 weeks [107]. The rate of mononuclear-cell division is elevated in early pregnancy and in chromosomally abnormal fetuses, probably as a consequence of higher number of circulating haemopoietic precursors. Hematopoietic and progenitor stem cells is found in both the fetal blood and liver [83], but the fetal liver acts as a richer source of primitive hematopoietic progenitor cells than the fetal blood. The fetal red blood cells, white blood cells, and platelet counts all increase with gestation, from weeks 8 to 17, reflecting hematopoietic development. During gestation, normoblasts decrease dramatically. The number of circulating and hepatic T lymphocytes increases before 13 week of gestation, reflecting thymic maturation. The fetal liver contains fewer T lymphocytes than the fetal blood (2.5 % vs. 18.6 %) and more CD34+ hematopoietic stem and progenitor cells (17.5 % vs. 4.3 %). At an early

gestational age (21–22 weeks) fetal blood has a higher concentration of primitive hematopoietic progenitor cells (CD34+/CD38− cells) than umbilical cord blood at full term (39–40 weeks) [82]. The cord blood cytokine-receptor network, consisting of IL-1, IL-2, IL-12, IFN-γ and TNF-α is biased towards anti-inflammatory activity in fetuses [41]. Endothelial progenitor cells derive mainly from the monocyte/macrophage-containing CD34- mononuclear cells and only in part from the hematopoietic stem-cell-containing CD34+ mononuclear cells [92].

Cytokine Profile of Fetal Pre-immunocytes

Increased IL-2γ receptor expression in cord-blood mononuclear can mediate the prevention of neonatal infection [94]. The mononuclear cells from neonatal blood produce less IL-10 than adults while the primary cells of origin and the regulatory mechanisms may differ in adults [57]. Enhanced IL-6 and decreased IFNγ production by the cord-blood mononuclear cells are detectable hallmarks of newborns population with the high-risk allergy population [64]. The fetal peripheral blood mononuclear cells exhibit mitogenic responses to allergenic stimuli during gestation [54].

Neonatal monocytes widely differ from adult monocytes in cytokine-expression profile [43]. Following exposure to lipopolysaccharide fetal monocytes produce more IL-8 and less TNF-α. In neonatal sepsis caused by *Streptococcus agalactiae*, a major cause of severe infection in newborns and pregnant females, different cytokine expression patterns (IL-6, IL-1 beta, and IL-12p40) have been found in the cord-blood mononuclear cells [6] compared to *Escherichia coli* lipopolysaccharide, where TNFą, IL-6, and IL-8 appear almost simultaneously. The human monocyte response to *S. agalactiae* results in the production of TNF-ą, and delayed appearance of IL-6, and IL-8 [61]. The lymphocyte response to *S. agalactiae* is manifested by IFNγ and IL-12 secretion, which the *E. coli* lipopolysaccharide fails to produce. This suggests an important role for TNFą, IFNγ, and IL-12 in *S. agalactiae* pathogenesis and/or immunity. Up-regulation of macrophage migration inhibitory factor (MIF) in the intervillous blood is found in placental malaria in pregnant women. MIF may play a role in immune responses to malaria during pregnancy by virtue of its ability to activate macrophages and to overcome the immunosuppressive effect of glucocorticoids [16]. A consistent pattern of MIF expression is found in the syncytiotrophoblasts, extravillous trophoblasts, intervillous blood mononuclear cells and amniotic epithelial cells [15]. Significantly higher levels of MIF expression is exhibited in the amniotic epithelial and intervillous blood mononuclear cells from infected placentas compared to that in uninfected placentas.

Development of Macrophages and Dendritic Cells

At 4–6 weeks of age there are found two populations of cells with a dendritic/macrophage structure in the yolk sac and mesenchyme and also prehaematopoietic liver at 5 weeks of gestation. The major population of yolk sac macrophages is MHC class II-negative, and there is a minor population that is MHC class II-positive [52]. MHC class II-negative cells appear in the thymic cortex, in the marginal zones of lymph nodes, in the splenic red pulp, and in the midst of erythopoietic activity in the bone marrow. A few MHC class II-positive cells are seen in the liver at 7–8 weeks of gestation, the lymph nodes at 11–13 weeks of gestation, and the T-cell areas of the developing thymic medulla by 16 weeks of gestation, whereas thymic epithelium expresses class II at 8–9 weeks [52].

MHC class II-positive cells also are present in the skin, gastrointestinal tract, and hepatic systems. The number of hepatic sinusoidal macrophages (Kupffer cells) is low in early gestation (17 weeks) but increases to nearly adult levels in the neonatal period. By 6 weeks of intrauterine development the blood flow to the liver passing through the left umbilical vein comes directly

from the placenta, providing a rich nutrient supply to these cells [19]. By 6–7 weeks of gestation HLA-DR+ Langerhans cells are detectable in the skin. Langerhans cells migrate into the epidermis during the first trimester and resemble the adult phenotype by the second trimester [33]. There are MHC class II-positive cells in unidentified cell type in the lamina propria of the fetal gut as early as 11 weeks of gestation [67]. Cord-blood dendritic cells express relatively poor accessory function [47]. Umbilical cord-blood dendritic cells have lower levels of ICAM-1 and MHC classes I and II than peripheral blood dendritic cells from adults.

T Cell Development

At 8–9 weeks of gestation CD7+ T-cell precursors from the fetal liver seed the thymus, 60 % of which are cytoplasmic CD2+ and only 4 % are cytoplasmic CD3+. From 9.5 weeks until birth, TCR β + cells increase to form over 90 % of the CD7+ population [13]. The prothymocytes by interacting with the stroma, proliferate, and differentiate with expression of the first T cell-specific surface molecules like CD2 and later, CD4 and CD8 [3]. At 12 weeks of gestation delineation of the thymic cortical and medullary regions occur, following which Hassall's corpuscles appear [8]. The most immature thymocytes are found in the subcapsular cortical region, and cells move into the deeper layers as they mature [9].

The early prothymocytes do not express CD3, the T cell receptor (TCR), CD4, or CD8 and are often referred to as "triple-negative thymocytes" [70]. The progeny continue to divide and rearrange their TCR genes, and since these cells express both CD4 and CD8, they are now called "double-positive" cells [58, 70]. They undergo positive selection by self-major histocompatibility complex (MHC) restriction. More than 95 % (nearly 50 million) of the cells die each day during this stage [70].

From 18 to 24 weeks of gestation, though the mesenteric lymph nodes have very few B cells or monocytes but a high percentage of CD45RA+ T cells, the fetal spleen at this time is invaded by equal numbers of T cells, B cells, and monocytes/macrophages [112]. Lymph-node and thymus T cells at these gestational ages express activation marker CD69. By 18 weeks of gestation T cells from fetal spleen have adult levels of CD3, CD4, CD8, CD2 and CD11a making the spleen fully immunocompetent, with sufficient accessory cells to ensure T-cell activation. But at this stage the mesenteric lymph nodes are deficient in accessory cells numerically or functionally.

Between 6 and 9 weeks of gestation prior to being detectable in the thymus rearranged TCR δ genes are first seen in the liver and primitive gut [71]. Though early in development the thymic and gut γ/δ T-cell repertoires overlap but they diverge and become non overlapping during the second trimester [117]. The γ/δ T-cell population in the fetal liver is distinct from the thymus. At 20–22 weeks of gestation, 63 % of CD3+ cells are TCR α/β and 32 % are TCR γ/δ in the liver, which is considered to be a site of γ/δ T-cell development. Mature αβ T cells can be found in the periphery of the human fetus as early as 10–12 gestational weeks [29].

Neonatal human T cells proliferate in response to different antigens, like allergens [89, 106, 111, 113], auto antigens [118], and parasite antigens [31, 81]. Mounting of an antigen-specific response of neonatal T and B cells is indicated by presence of antigen-specific IgE in umbilical cord blood [56].

Treg Development

In the fetal thymus, double-positive cells initiate expression of CD25, GITR, CTLA4 and CD122 during their transition from the CD27− to the CD27+ stage. Moreover, CD4 + CD25+ fetal thymocytes already have the potential to suppress proliferation of CD25- cells. After leaving the thymus, FoxP3 + CD4 + CD25+ Treg enter the fetal lymph nodes and spleen, where they acquire a primed/memory phenotype [21].

Development of B Cells

As early as 8 weeks of gestation Pro- (CD24+/surface IgM-negative) and pre-B cells (cytoplasmic IgM+/surface IgM-negative) can be detected in the fetal liver and omentum. The percentage of pre-B cells decreases during weeks 13–23 in the omentum though they are similar to fetal liver over 8–12 weeks gestation [101]. B-cell development in the omentum is transitory and become detectable in the spleen at 13–23 weeks of gestation. At 15 weeks of gestation CD5+ B cells can be found in the human peritoneal cavity and pleural cavity [80]. In the fetal circulation percentage of CD5+ B cells (B-1 B cells) is higher than the adult, and declines with increasing gestational age [10, 110]. CD5+ B cells are largely T-independent and produce polyreactive antibodies which have a role in the primary immune response. This immune mechanism form a part in the first line of defence, which is a necessary function in the newborn.

The pre-B cell, is a large lymphoid cell containing scanty intracytoplasmic μ heavy chains and lack surface immunoglobulins (sIg) and intracytoplasmic light chains [34, 42, 90]. There were twice as many pre-B cells as B cells in the livers of 7–14 week old fetuses [34, 55]. Although both small and large pre-B cells were seen in the liver and BM at all times of gestation investigated, the small pre-B cells by far outnumbered the large ones in both organs [59]. At 8 weeks of gestation, liver pre-B cells express the cytoplasmic μ chain, and by 10–12 weeks, surface IgM is expressed on liver B cells. In the livers of 8 week old fetuses, more than 90 % of the total pre-B plus B cell population consisted of pre-B cells; the relative number of liver pre-B cells gradually decreased with increasing gestational age and, after the 14th week, B cells outnumbered pre-B cells [35].

From 13 weeks of gestation surface IgD is detectable and CD24 expression precedes μ-chain expression and is retained throughout differentiation into adulthood. Liver B cells are negative for CD21 and CD22 though they express CD20 [10]. B cells strongly express IgM+ which are detectable in the lymph nodes from 16 to 17 weeks and spleen at 16–21 weeks [10]. From 17 weeks of gestation primary nodules develop around the follicular dendritic cells of the lymph nodes. At 16–20 weeks of gestation B cells are abundant in the bone marrow. B cells in the spleen are diffusely distributed at 22 weeks, and then form primary nodules around 24 weeks; this is later than seen in lymph nodes. B cells positive for CD19, CD20, CD21, CD22, HLA-DR, IgM, and IgD emerge into the peripheral circulation at 12 weeks of gestation [110].

Immunoglobulin Production

At 10 weeks of gestation early IgG and IgM synthesis occurs primarily in the spleen in large amounts, and is maximal at 17–18 weeks of gestation. Between 5.5 and 22 weeks, serum IgG level slowly increase with greater increase in 26 weeks, and finally a very significant increase at birth [37]. Throughout gestation IgG crosses the placenta, the transfer rate rising markedly from 20 weeks, and is maximal from 32 weeks of gestation [69, 84]. At 11 weeks of gestation IgE synthesis was observed in fetal liver and lung, and by 21 weeks in the spleen [74].

Neonates have very low serum IgM, IgA and IgE levels, and the IgG present is essentially of maternal origin. Surface IgM and CD79 (signal transducer for membrane Ig and necessary for all IgM functions) are elevated on cord-blood B cells compared to the adult. But similar levels of CD19, 21, 22, and 81 are expressed in cord and adult B cells, although CD32 is lower on cord B cells [66].

Newborns of mothers vaccinated with tetanus toxoid during pregnancy have specific antibody of the IgM class in their serum [36]. Umbilical cord mononuclear cells collected at birth at full term exhibit antigen-specific reactivity to allergens, including those of house-dust mite and cow's milk [12, 81, 83, 107]; parasite antigens such as those of *Plasmodium* spp. [115] and *Schistosoma* spp. [92]; and autoantigens, including myelin basic protein [41].

Mucosal Immunity

IgA and IgM are important in the first line of defence. Occasional IgM- and IgA-producing cells were observed in the fetal parotid gland (20–40 weeks), but cells producing D, G, or E isotypes were not seen [108]. The IgA1 subclass mostly J-chain-positive predominates. Amylase, lysozyme, and lactoferrin were detectable early fetal life.

Duodenal expression of secretory component, classes I and II is seen and IgA-, IgM-, and IgG-producing cells are detectable from 24 to 32 weeks of gestation. A population of MHC class II-positive cells exists in the lamina propria from 11 weeks of gestation [53], and T cells are found at this site from 12 to 14 weeks of gestation [30]. From the second postnatal week, intense expression of epithelial HLA-DR, secretory component, and IgA is seen, reflecting modulation by environmental factors [108].

Mucosal surfaces have an important role in the development of allergic responses and disease immune responses. Prior to birth both the skin and gastrointestinal tract are relatively immunologically mature, whereas the airways show little evidence of population by haematopoietic cells; an influx is seen during the first week postnatally [102]. Allergic disease is first manifest in the gut and skin while airways inflammation appear later in infancy/childhood. This may be due to developmental delay in the airways.

Development of Eosinophils

Eosinophil granulopoiesis occurs in the fetal liver, at 5 weeks in the hepatic laminae [100], and after 20 weeks of gestation, they appear in the portal areas. There are very few studies on either the phenotype or function of fetal and neonatal eosinophils. Interestingly, fetal eosinophils (23–34 weeks of gestation) have adult levels of L-selectin, while newborns have less L-selectin on their eosinophils than those of the adult [98, 99].

Mother–Placenta–Fetus: Relation to Infection and Immunity

Neonates with placental infections have very high circulating levels of inflammatory cytokines, such as IL-1, IL-6, IL-8 and TNF-α, which has been termed as fetal inflammatory response syndrome (FIRS) [22, 68, 91]. These cytokines have been shown to affect the CNS and the circulatory system [14, 24, 68]. Fetal morphologic abnormalities were found in animal models leading to ventriculomegaly and hemorrhages, which were due to fetal pro-inflammatory cytokines such as Il-1, TNFα, MCP-1, MIP1-β and INF-γ. The presence of FIRS increases the future risk for autism, schizophrenia, neurosensorial deficits and psychosis induced in the neonatal period. The presence of FIRS increases the future risk for autism, schizophrenia, neurosensorial deficits and psychosis induced in the neonatal period [38, 72, 96]. An inflammatory response in the placenta, altering the cytokine balance in the fetus, may affect the normal development of the fetal immune system leading to anomalous responses during childhood or later in life. A significant impact on later vaccine responses may result from antenatal infections. Surviving infants with placental malaria may suffer adverse neurodevelopmental stages and may have abnormal responses to a later parasitic infection [62]. In all these cases the parasite did not reach the placenta, but the inflammatory process in the placenta affected the normal fetal development [23].

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Part VII

Fetal Hepatic Development: Up to Second Trimester

Growth and Development of Human Liver and Its Future Implications

25

Gopal Krishna Dhali and Gurubasava Lakamaji

Anatomy of Liver

Liver is the largest gland in the body comprising ~5 % of body mass in mammals. It performs important metabolic, endocrine and exocrine functions. Human liver normally weighs 1.44–1.66 kg and is located in right upper quadrant of abdomen. Two major types of cells populate the liver lobes; parenchymal and non-parenchymal cells. Eighty percent of the liver volume is occupied by parenchymal cells i.e. hepatocytes. Non-parenchymal cells constitute 40 % of the total number of liver cells but only 6.5 % of its volume. Sinusoidal hepatic endothelial cells, Kupffer cells and hepatic stellate cells are non-parenchymal cells.

The microscopic structure is conceptualized in several ways, the two most common being the acinus and the lobule. The acinus is a unit that contains a small portal tract at the center and terminal hepatic venules at the periphery. It is the smallest functional unit and is divided into zones 1, 2, and 3, wherein zone 1 surrounds the portal tract and zone 3 surrounds the hepatic venule. Alternatively, the traditional concept of lobule may also be used, in which the central structure is the terminal hepatic venule ("central vein") and the periphery is delineated by portal tracts [1] (Fig. 25.1).

The microscopic anatomy of the liver consists of clusters of cells called lobules. Each lobule, measuring about 1 mm in diameter, consists of numerous cords of hepatocytes, that radiate from central vein toward a thin layer of connective tissue that separates the lobule from other neighboring lobules. The cords of liver cells are one cell thick and are separated from one another on several surfaces by spaces called sinusoids, compartment between endothelial cells and hepatocytes that contains the microenvironment for exchange between blood and hepatocytes is called as 'Space of Disse' [2, 3].

The portal triad at periphery of the lobule contains three important structures, the portal vein, the hepatic artery and the bile duct. These structures lie in a network of connective tissue and are surrounded on all sides by hepatocytes. The ring of hepatocytes abutting the connective tissue of the triad is called the periportal limiting plate [1, 2].

Physiology of Liver

Liver performs many physiologically important functions.

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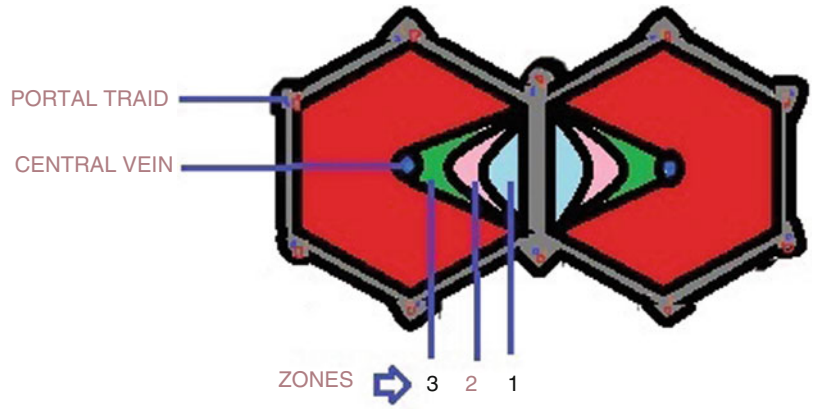

Fig. 25.1 Microscopic anatomy of liver

Bile consist of bile acids, bile pigments, and lipid components. About 500 mL is secreted per day. Some of the components of the bile are reabsorbed in the intestine and then excreted again by the liver (enterohepatic circulation). It has important role in digestion and absorption of fats, and serves as route for lipid-soluble waste products excretion [4].

The various nutrients absorbed in GI tract are transported to liver through portal vein which are processed by hepatocytes before they enter systemic circulation. Liver plays key roles in carbohydrate metabolism such as glycogen storage, gluconeogenesis, and regulate blood glucose levels, it is also a key organ involved in cholesterol and ammonia metabolism [4, 5].

Liver inactivates and detoxifies various endogenous and exogenous substances. It is mediated in their first stages by cytochrome P-450 enzymes expressed in hepatocytes. These convert (xenobiotics) and other toxins to inactive, less lipophilic metabolites [4, 5].

Liver synthesizes albumin which accounts for the majority of plasma oncotic pressure. Other substances that are synthesized by liver are transport proteins for steroids and hormones, clotting factors, and various acute-phase proteins [4].

Introduction to Development of Liver

GI tract is principally derived from endoderm with contribution from other mesoderm and ectoderm. Around 16th day of human development. Endoderm-lined yolk sac cavity is formed due to horizontal and median folding of embryo into primitive gut. In the cephalic and caudal parts of the embryo, the primitive gut forms a blind-ending tube, the foregut and hindgut, respectively. The middle part, the midgut, remains temporally connected to the yolk sac by means of the vitelline duct.

The liver and biliary system arise from cells of the ventral foregut endoderm; the parenchyma of the liver (cords of hepatocytes and branched tubules of bile ducts) intercalates within the tissue of the septum transversum and the plexus of vitelline vessels, accounting for the overall architecture observed in the adult (plates of hepatocytes, which are endoderm derived, surrounded by vascular sinusoids, which are mesoderm derived)., the ventral bud of endoderm that gives rise to liver grows into the ventral mesentery, thus becoming surrounded by mesodermal tissue. The portion of the mesentery into which the liver grows is involved in the formation of the septum transversum. Thus the developing liver becomes enclosed in the septum. The mesodermal tissue gives rise to the fibrous capsule of Glisson and to the small amount of connective tissue within the gland. This developmental story is a complex process involving interaction between cells and various growth factors most important of this is BMP, HNF etc.

The first molecular evidence of liver development of liver is the expression of albumin, transthyretin, and alpha-fetoprotein in a region of the ventral endoderm. In vivo DNA/protein analyses

have shown that the albumin gene promoter is bound by Forkhead box (Fox) A and GATA-4 transcription factors at a stage that precedes transcription of the gene. FoxA and GATA factors decompact chromatin, binding of such factors opens chromatin to provide access to additional transcription factors resulting in the transcriptional activation of albumin [6, 7].

Hepatocyte nuclear factor (HNF)-1 stimulates expression of FoxA1 and FoxA2 in the prehepatic endoderm; in its absence, the endoderm cells lack competence and hepatic specification is impaired. Thus, HNF-1, FoxA1, FoxA2, and GATA-4 are critical initiators of liver development [8].

Extracellular signals act in specific endodemal cell lines and induce their specification to a hepatic fate. Fibroblast growth factors (FGFs) secreted by the cardiogenic mesoderm is one of such important factor. Hepatic gene induction by FGF is mediated by a mitogen-activated protein kinase signaling cascade [9] and is dependent on low concentrations of FGF [9].

Septum transversum also involved in regulation of liver development it produces bone morphogenetic protein (BMP)-2 and BMP-4, which along with FGF involved in hepatic gene expression. BMP saction is probably mediated by involvement of GATA-4 transcription factor [10].

Wnt signaling has got important role in liver development. When the primitive gut becomes patterned along its anteroposterior axis, Wnt signaling must be repressed anteriorly to maintain foregut identity and to allow subsequent liver development which is achieved by secretion of Wnt inhibitors by endoderm [11].

Liver Bud Formation

When endoderm cells have been specified, a diverticulum forms from the primitive gut. This occurs day 22 in humans. The liver diverticulum is lined by endoderm cells, which at this stage are called hepatoblasts, that become columnar and undergo a transition to a pseudostratified epithelium. The hepatoblasts proliferate and form a tissue bud delineated by a basement membrane that contains laminin, collagen IV, nidogen, fibronectin, and heparan sulfate proteoglycan. The hepatoblasts then leave the endoderm epithelium, migrate through the basement membrane, and invade the septum transversum [44].

A network of transcription factors controls the onset of liver development. Studies of knockout mice have shown that the hematopoietically expressed homeobox factor (Hex) promotes interkinetic nuclear migration and hepatoblast proliferation GATA-6 is required to maintain the differentiation state of the hepatoblasts. absence of either factor, liver development is arrested soon after budding. In prospero-related homeobox 1 (Prox-1)—deficient mice, hepatoblasts remain abnormally clustered in the liver bud. It has been proposed that the lack of Prox-1 leads to a failure of the hepatoblasts to delaminate from the liver bud and to migrate in the septum transversum, resulting from overexpression of E-cadherin, which holds the cells together by cell-cell contacts. Other factors involved in development are, HNF-6, Onecut (OC)-2, Tbx3 [44, 45].

There is necessity of continuous interactions between hepatoblasts and adjacent mesodermal tissues. When the activity of metalloproteases produced by the hepatoblasts and mesenchymal cells is blocked, migration of the hepatoblasts through the basement membrane is inhibited.

Also, the endothelial cells that surround the liver bud promote hepatoblast proliferation, as illustrated by the lack of liver expansion in mice with deficiencies in endothelial cell development.

Onset of Liver Development

The parenchymal cells of the liver i.e. hepatocytes are derived from the anterior portion of the endoderm. Mesothelial cells of septum transversum which are formed out of lateral plate mesoderm also have got important role to play in liver development. Coculture studies using chick embryos demonstrated that the developing cardiac mesoderm plays a crucial instructive role during the induction of hepatic cell fate. These inductive signals were found to be members of the fibroblast growth factor family, since FGF1 and FGF2 could substitute for cardiac tissue in inducing the onset of Albumin expression, a characteristic marker of

hepatic cell fate, in explants of mouse endoderm. FGF-mediated specification of hepatic cell fate is concentration dependent and this appears to be controlled by the position of the endoderm relative to the heart [7, 12].

The induction of hepatic gene expression by FGF is controlled through activation of the MAPK pathway. FGFs expressed on cardiac mesoderm during embryogenesis include Fgf1, Fgf2, Fgf8, and Fgf10. Interestingly FGF signaling in liver development is evolutionarily conserved, with FGFs displaying hepatogenic properties in Xenopus, chick [13, 14].

GATA4, a zinc finger transcription factor, is also expressed in septum transversum mesenchymal cells that surround the liver bud and is required for the liver bud to expand. It regulates expression of the secreted bone morphogenetic protein, BMP4, which, like GATA4, is highly expressed in the septum transversum mesenchymal cells at an early stage of development. Analyses of Bmp4–/– mouse embryos also revealed a delay in expansion of the liver bud and addition of BMP inhibitors were found to block hepatic specification in vitro [15, 16].

Zaret and colleagues worked on a model whereby TGFb signaling acts to restrict endoderm specification as cell movements position progenitors within the appropriate inductive environments. In This proposed model, TGFb acts as a developmental timer to ensure that the endoderm retains hepatic competency and is prevented from inappropriately differentiating.

The WNT signaling pathway has also been implicated during the onset of hepatic development, the contribution of WNT signaling appear complex. WNT signaling makes different contributions depending on the developmental stage. At early somite stages WNT signaling acts in the posterior endoderm to repress expression of Hhex, an essential transcriptional regulator of hepatic development. Repression of WNT signaling by expression of WNT antagonists in the anterior endoderm is, therefore, required to relieve repression of Hhex in the anterior endoderm and so facilitate commitment of the endoderm to a hepatic fate. In contrast to the repressive effects of WNTs at early somite stages, following specification, WNT signaling appears to promote hepatogenesis. This factor is expressed in the lateral plate mesoderm that is positioned adjacent to the endoderm [11].

Transcriptional Regulation of Hepatogenesis

Multiple transcriptional factors act in association with cell signaling molecules to regulate development of liver. Albumin expression is seen in early stage of development by nascent hepatic cells. DNA footprinting analyses revealed that FoxA and GATA4 both of which are expressed in the anterior endoderm, bind to the Albumin enhancer and interact with their respective binding sites in the context of compacted chromatin and results in displacement of linker histone H1 and repositioning of nucleosomes and subsequently albumin synthesis [9]. But, hepatocyte differentiation and control of gene expression, following specification of the liver progenitors, seems to be independent of FoxA1/A2.

Hepatocyte growth factor (HGF) is expressed by the septum transversum, the endothelial cells, and the hepatoblasts. Its receptor, c-met, is found at the surface of hepatoblasts, where it initiates a signaling cascade mediated by SEK1/MKK4 and possibly by c-jun. This cascade promotes hepatoblast proliferation, as indicated by the reduced proliferation of cells in mice with disruptions in genes that encode HGF, c-Met, SEK1/MMK4 [46].

The transforming growth factor (TGF) B Smad2/Smad3 pathway also stimulates proliferation, because mice with heterozygous mutations in the Smad2 and Smad3 genes showed liver hypoplasia. HGF and TG pathways functionally interact. Cultured explants from livers deficient in Smad2 and Smad3 had reduced expression of A 1-integrin, but this reduction was rescued by the addition of HGF to the medium. It is likely that the HGF and TGF b pathways converge on a1-integrin expression, which is necessary for proliferation [47].

Hepatoma-derived growth factor (HDGF) is produced by fetal hepatoblasts and stimulates their proliferation when they are cultured in vitro. The expression of HDGF remains high at the hepatoblast stage and is extinguished when the hepatoblasts mature to hepatocytes [48].

Studies in mouse embryos have found that the homeodomain transcription factor HNF1b is also essential for hepatic specification. In embryos lacking HNF1b, the liver bud fails to express any markers of hepatic parenchymal cell progenitors and the level of mRNAs encoding the FoxA factors was severely reduced [8].

The homeobox transcription factor Hhex regulates proliferation and positioning of the ventral endoderm within the cardiogenic field. In the absence of Hhex, mutant mouse embryos initiate hepatic specification but fail to complete liver bud morphogenesis, resulting in hepatic structures that lack a parenchymal cell component. It is suggested that GATA4 and/or GATA6 may be involved in transactivating the Hhex promoter [17].

The prospero-related homeobox transcription factor Prox1 also promotes hepatoblast proliferation and migration from the primary liver bud. Although the mechanism through which Prox1 controls hepatoblast migration is unclear, the mutant hepatoblasts were found to maintain high levels of E-cadherin and failed to degrade the basal matrix surrounding the liver bud. Recent work has shown that the T box transcriptional repressor Tbx3 may act upstream of Prox1. In control embryos the expression of key regulators of hepatocyte differentiation including Hnf4a and c/EBPa are strongly expressed in the migrating hepatoblasts, whereas expression of transcription factors that primarily control cholangiocyte fate, such as HNF6 and HNF1b, are found to be at very low levels. In Tbx3 mutant mice expression of Hnf4a and c/EBPa is lost while levels of HNF6 and HNF1b are increased suggesting that Tbx3 normally promotes a hepatocyte fate and represses a cholangiocyte fate [18].

TNF stimulates a signaling cascade that activates the transcription factor nuclear factor kB (NF-kB). NF-kB is composed of the subunits p50 and p65/RelA and is activated by IkB kinases. Mice deficient in IkB kinase k or IkB kinase gamma, showed massive apoptosis in the liver. Interestingly, the dramatic phenotypes of IkB kinas -null mice were rescued by the additional inactivation of the TNF receptor, indicating that NF-kB protects hepatoblasts against TNF-induced apoptosis.

Thus from above discussion it is obvious that formation of ventral bud and subsequent morphogenesis is regulated by multiple factors which are intricately connected and complex.

Seggregation of Hepatobiliary Lineage

Concept of the cell fate decision applies, in theory, to hepatoblasts that have reached a developmental stage at which they become committed either to the hepatocyte lineage or the cholangiocyte lineage. Hepatocytes have a number of specific markers that are already present at the endodermal stage, so the onset of hepatocyte differentiation cannot be accurately defined. The analysis of mice with a disruption in the gene encoding the transcription factor HNF-4 showed deficient expression at E12 of a number of hepatocytespecific proteins such as apolipoproteins or albumin, suggesting that HNF-4 is critical for hepatocyte fate determination [49].

Another way to determine the timing of lineage segregation is by analysis of expression of biliary markers. For example, the expression of the intermediate filament proteins cytokeratin 19 and 7 is considered a sign of biliary differentiation. However, because cytokeratin 19 is expressed at low levels in hepatoblasts and at increasing levels in differentiating cholangiocytes SRY-related HMG box transcription factor 9 (SOX9) is present in endodermal cells that line the hepatic diverticulum, but its expression disappears when the cells start to invade the septum transversum. SOX9 becomes reexpressed at later developmental stages, it is restricted to biliary cells. The expression of SOX9 at can therefore be

considered the earliest indication biliary lineage differentiation.

Hepatocyte Maturation

Cells that have differentiated to the hepatocyte lineage undergo a process of maturation that consists of the progressive acquisition of morphology and physiologic functions. Several microarray analyses of gene expression at various stages of liver development support the view that maturation is a process that extends throughout development, even after birth. The transcription factors that control hepatocyte maturation nicely illustrate the concept of a "dynamic transcriptional network" [50].

Metabolic and cellular functions of hepatocytes is regulated by various transcriptional activators and related cofactors. In addition to the expression of hepatic genes throughout the parenchyma, expression of some genes is restricted to zonal regions that are often related to the position of the portal triad (periportal) or central veins (pericentral/perivenous) which lead to diversity of metabolic functions of hepatocytes depending on their location [18].

WNT/b-catenin signaling pathway are found to be involved in controlling the positional identity of hepatocytes within the liver lobule. b-catenin is important for zonal gene expression in perivenous hepatocytes and this activity is antagonized by adenomatous polyposis coli (APC) in the periportal regions. In studies involving mice that lack HNF4a in hepatocytes found an increase in periportal expression of a subset of perivenous expressed genes. HNF4a was found to directly interact with the glutamine synthetase enhancer suggesting that HNF4a inhibits expression of pericentral mRNAs in periportal hepatocytes possibly by recruiting the histone deacetylase protein HDAC1. Activation of the Wnt pathway converted hepatocytes that exhibited a periportal character to those that expressed perivenous markers. In response to activation of Wnt signaling, a transcription factor activated by b-catenin called LEF1 was found to physically interact with HNF4a [19–21].

Interestingly expression of several genes, including alphafetoprotein, H19, and Glypican-3, is seen in fetal hepatocytes but reduced in fully differentiated cells. This is often reversed as hepatic cells become cancerous. Recent studies of the alphafetoprotein promoter have revealed an important role of transcriptional repressors in controlling the transition of the gene expression profile within hepatocytes from fetal to an adult. Alphafetoprotein regulator 1 (Afr) was genetically defined as a locus that conferred a high level of AFP expression. Recently, genetic mapping studies revealed that the increase in AFP expression in adult livers was a consequence of a retrovirus insertion into the Zhx2 gene. Zhx2 in mediating repression of Afp expression, a direct interaction with the Afp promoter has not been identified, raising the possibility that Zhx2 regulates Afp indirectly. Zhx2 also represses expression of H19 and glypican-3 [22].

Hepatic Vasculature Development

The fetal liver is in contact with two major venous systems, the umbilical veins and the vitelline veins. The vitelline veins participate in the formation of the efferent venous system of the liver. The umbilical vein is the major afferent vessel in the fetal liver, but its presence is transient and it disappears after birth. When the umbilical vein collapses, the portal vein replaces it as the major afferent vein. Hepatic artery development occurs later than venous development. It starts to form along the intrahepatic portal vein within the parenchyma and gradually extends toward the periphery. The current model suggests that intrahepatic arterial development in humans is driven by the ductal plate that forms at the same stage and is a source of VEGF [23, 24].

Blood that is carried into liver enters into specialized vascular channels known as sinosoids. They are important route of exchange of various substances. Sinusoids are the first blood vessels to form during hepatogenesis, they develop by angiogenesis from existing vessels in the septum transversum mesenchyme. As development progresses, the sinusoidal endothelial cells gradually

adopt the functional and structural characteristics of mature sinusoids. Although the molecular mechanisms that control growth and maturation of sinusoidal endothelial cells are not well defined, several studies support a role for Wnt signaling in their proliferation and differentiation. In particular, Wnt2 was shown to be expressed in rat hepatic sinusoidal endothelial cells and could increase their proliferation through activation of canonical b-catenin signaling. When Wnt2 levels were depleted it resulted in a decrease in expression of VEGF receptor 2 in rat sinusoidal endothelial cells. This implies that the autocrine activity of Wnt2 cooperates with VEGF signaling to control sinusoidal endothelial cell growth in the liver [24, 25].

Hematopoiesis

Soon after the liver progenitors invade the surrounding mesenchyme, the fetal liver is colonized by hematopoietic progenitors and transiently becomes the principal hematopoietic organ. Immature hepatic progenitor cells can generate an environment that supports hematopoiesis; however, when hepatic progenitor cells are induced to differentiate to a mature form, this is abolished leading to movement of hematopoietic stem cells from the fetal liver to the adult bone marrow, during this general timeframe. It involves activation of Wnt signaling pathways by progenitor cells. Hematopoietic cells within the fetal liver express the cytokine Oncostatin M (OSM) which enhance differentiation into hepatocyte, thus there is a dynamic interplay between the blood and parenchymal compartments within the fetal liver that controls the timing of both hepatogenesis and hematopoiesis [26].

Stellate Cells

Stellate cells to perform many important function such as they store vitamin A and to modulate hepatic microcirculation in response to endothelin signaling. After injury to liver cells, stellate cells can also become activated to adopt a myofibroblast character and chronic activation of stellate cells leads to liver fibrosis.

Initial research suggested that these cells could be of endodermal, neural crest, or mesenchymal origin. Recent studies have suggested that mesothelial cells derived from the proepicardium and septum transversum mesenchyme could give rise to both endothelial and stellate cells [28].

Hepatic stellate cells and derivatives of the septum transversum mesenchyme may contribute toward the fate of other hepatic cell lineages. The homeobox protein Hlx is expressed in the septum transversum and visceral mesenchyme. In Hlx–/– mouse embryos livers were severely hypoplastic containing only 3 % of the cells found in control liver, this could be due to regulation of expression of mitogens or growth factors by Hlx [27].

Kupffer Cells

Kupffer cells are resident macrophages on the surface of hepatic sinusoidal endothelial cells. They represent 15 % of the liver cell population and 50 % of resident macrophages in the body. There is no clear report on the role of Kupffer cells in liver organogenesis. However, some data suggest that Kupffer cells or their progenitors may be involved in maturation of erythrocytes during fetal liver hematopoiesis. They could be possibly of mesenchymal origin [29].

Development of Extrahepatic Bile Ducts

In the embryo approximately 5 mm in length, the hepatic diverticulum shows a protruding bud in its distal part. Some investigators accordingly distinguish in the hepatic diverticulum a cranial part (pars hepatica) and a caudal part (pars cystic).

The caudal bud or pars cystica grows in length and represents the anlage of the gallbladder, the cystic duct, and common bile duct (ductus choledochus). For up to 8 weeks of gestation, the extrahepatic biliary tree further develops through lengthening of the caudal part of the hepatic

diverticulum. At 29 days after fertilization, the gallbladder anlage is visible as a right anterolateral dilatation along the distal half of the hepatic diverticulum, with a cystic duct present at 34 days. At that stage, the gallbladder and cystic duct are provided with a lumen. Outpockings appear in the gallbladder wall in the 42-mm embryo; folds develop on the interior surface of the bladder at the 78-mm stage [30].

Myoblasts develop around the 30-mm stage, resulting in the establishment of all three layers of the wall of the future gallbladder: the mucosa, the muscular layer, and the serosa [31].

The pars cystica of the hepatic diverticulum begins initially from the anterior side of the future duodenum. Approximately the fifth week, the duodenum rotates to the right, so that the attachment of the developing common bile duct becomes displaced to its definitive position on the dorsal side of the duodenum [32].

In the 34-day embryo, the common hepatic duct is a broad, funnel-like structure in direct contact with the developing liver, without a recognizable left or right hepatic duct. During the fifth week, a rapid entodermal proliferation takes place in the dilated funnel-shaped structure above the junction of common bile duct and cystic duct; this proliferation gives rise to several folds, resulting in several channels at the porta hepatis. The distal portions of the right and left hepatic ducts develop from the extrahepatic ducts and are clearly defined tubular structures by 12 weeks of gestation. The proximal portions of the main hilar ducts derive from the first intrahepatic ductal plates. The extrahepatic bile ducts and the developing intrahepatic biliary tree maintain luminal continuity from the very start of organogenesis throughout further development [33].

Hes 1, the protein product of the Hes 1 gene is expressed in the epithelium of the extrahepatic bile ducts throughout their development. In vivo loss of Hes 1 (a transcription factor directly regulated by the Notch signalling pathway) results in agenesis of the gallbladder and hypoplasia of the extrahepatic bile ducts. The mRNA of the onecut transcription factor HNF6 is expressed in mouse liver from the onset of its development, and is detected not only in hepatoblasts but also in the extrahepatic bile ducts and the gall bladder primordium. In the mouse, development of the gallbladder further requires normal functioning of the forkhead box f1 gene (foxf1 gene). Haploinsufficiency of the Foxf1 transcription factor results in gallbladder malformation, with inadequate external smooth muscle layer, insufficient mesenchymal cell number [34, 35].

Intra Hepatic Bile Ducts

Seventh week as the time point for the first appearance of the intrahepatic bile duct system and describes a junction between the extrahepatic bile ducts and the earliest intrahepatic bile duct structure at the time of its first appearance in the liver hilum [36].

There are multiple theories of intra hepatic bile radical development. One theory maintains that the intrahepatic biliary tree is derived from ingrowth of the epithelium of the extrahepatic ducts. Another postulates that the entire intrahepatic biledraining system develops from hepatocyte precursor cells. A third theory combines elements of both of the first two. Most investigators favor the second hypothesis [37].

The hepatoblastic origin of the intrahepatic bile duct system received additional support from several studies that reinvestigated the embryologic development of the intrahepatic bile ducts with immunohistochemical techniques. These studies made use of immunohistochemical stains for cytokeratins, tissue polypeptide antigen, carcinoembryonic antigen, epithelial membrane antigen, and other markers for parenchymal and bile duct cell phenotypes [37].

Cytokeratins are the intermediate filaments of the cytoskeleton characteristic for epithelial cells. Different cytokeratins have been identified and catalogued. Normal adult human liver parenchymal cells express only the cytokeratins 8 and 18; intrahepatic bile duct cells express in addition the cytokeratins 7 and 19 and 20 [38].

Around the eighth week of gestation, the primitive hepatoblasts adjacent to the mesenchyme around the largest hilar portal vein branches become more strongly immunoreactive for their cytokeratins 8, 18, and 19. This layer of cells, surrounding the portal vein branches like a

cylindrical sleeve, is termed the ductal plate. Recent studies unraveled that biliary cell differentiation is induced in the fetal liver by a periportal gradient of activin/transforming growth factor-beta (TGFb) signaling, the extent of which is controlled by the inhibitory influence of HNF6 and the Onecut factor OC-2 [39].

Forkhead Box m1 transcription factor (Foxm1b) is also essential for normal intrahepatic bile duct cell differentiation, besides development of vessels and sinusoids. The ductal plates adjacent to the mesenchyme (the portal ductal plate layer) become duplicated by a second layer of more keratin-rich cells over variably long segments of their perimeter (the second or lobular ductal plate layer; During the following weeks, ductal plates also appear around the smaller portal vein branches at a distance from the hilum. In the meantime, the hepatoblasts not involved in ductal plate formation gradually lose cytokeratin 19, and by 14 weeks of gestation the future parenchymal cells are immunoreactive only for cytokeratins 8 and 18, the cytokeratin pair normally expressed in adult liver parenchymal cells [40].

From approximately 12 weeks of gestation onward, a progressive "remodeling" of the ductal plates takes place, starting again in the earliest ductal plates around the larger portal vein branches near the hilum. Over short segments of the perimeter of the double-layered ductal plates, a "tubular" dilatation occurs of the slit-like lumen, lined now by taller keratin-rich cells. These cells acquire epithelial membrane antigen and lose their biliary glycoprotein I. The "tubular" parts of the ductal plate become incorporated into the mesenchyme surrounding the portal vein (the future portal tract) by ingrowth of mesenchyme between the lobular ductal plate layer and the hepatoblasts. The tubules incorporated into the portal mesenchyme are the future portal ducts; they remain connected, however, to the ductal plate and its adjacent parenchyma by thin epithelial channels, assuring continuity between the portal ducts and the already developed canalicular network located between the primitive hepatocyte precursor cells [41].

By 20 weeks of gestation, weak immunoreactivity for cytokeratin 7 appears in the cells of the developing ducts, again appearing first in the older ducts near the hilum. The immunoreactivity for cytokeratin 7 gradually increases and extends into more peripheral ducts, to reach the level of immunoreactivity observed in ducts of the adult liver at approximately 1 month after birth. At the time of birth, the most peripheral branches of the intrahepatic biliary tree are still immature: The finest portal vein radicles still are surrounded by ductal plates, which require an additional 4 weeks after birth to develop into small portal ducts. This finding indicates some degree of immaturity and incompleteness of the intrahepatic bile duct system in the neonate and may explain the lower ratio of the total number of bile ducts to the total number of portal tracts in premature infants [42].

The factors determining the developmental fate of the bipotential hepatoblasts remain incompletely known. There is evidence that components of the portal mesenchyme are crucial for inducing the phenotypic shift into bile duct-type cells in the layers of the ductal plate Several components, including laminin, fibonectin, collagen types I and IV, expression of corresponding cellular integrins, catenin, and N-CAM have all been shown to be involved. The process of intrahepatic bile duct development comprises schematically the following components:

(a) A gradual phenotypic change of the hepatoblasts toward bile duct type cells;
(b) A remarkable remodeling of the tridimensional structure of the ductal plate; and
(c) An ongoing further maturation of the remodeled, tubular ducts [43].

The remodeling of the ductal plate involves epithelial changes—construction of new epithelial structures (by proliferation) and the simultaneous deletion of other parts (by apoptosis), mesenchymal influence, and determination by the portal vein. Autocrine stimulation of ductal plate cell proliferation is suggested by the immunohistochemical positivity for transforming growth factor-a, hepatocyte growth factor, and parathyroid hormone-related peptide. Apoptosis in the remodeling ductal plate is indicated by positive histochemical terminal deoxynucleotidyl transferase mediated bio-deoxy UTP nick end labeling (TUNEL) staining and expression of Fas, C-myc, and Lewisy.

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Embryonic Development of Liver and Its Future Implications

26

Abhijeet Chaudhuri

Introduction

The growth and maturation of human liver, as an organ, keeps pace with the structural and metabolic needs of the developing fetus and is responsive to the highly dynamic milieu that fetal growth entail. Understanding aspects of this development, including its' regulation, is an intriguingly important matter in molecular and cell biology. Aberrations in development can give rise to human diseases that have health impact. More importantly, liver is the abode for a variety of stem cells – both for the bone marrow derived that are pluripotent and home to the liver as well as the hepatic progenitor cells (HPC) that are more differentiated, oval cells – playing roles in hepatocyte regeneration and response to injury. Development of the liver entail organization of the micro and macro elements of this organ in a simplistic view. More consequential of understanding this mechanism would be to serve as a reserve source for replenishment and refurbishment of the organ in times of repair and replacement during liver injury and cell loss. Everyday, we are learning this with greater precision and with the advent of molecular tools as well as refinements in cell biology, understanding of normal and abnormal development of the liver is fairly robust now. This has implications that extend far beyond just enrichment of the knowledge. Understanding the process of development might enable us to create new cell therapies- growing tissues ex vivo for use in transplantation or for coaxing cells in vivo to acquire characteristics that can restore function in disease states.

Adult Liver Architecture/ Morphology

Grossly adult liver measures 12–15 cm coronally and 15–20 cm transversely. The median liver weight is 1,800 g in men and 1,400 g in women The adult liver weight is between 1.8 and 3.1 % of body weight in 80 % of individuals [1, 2]. While liver weights in fetuses and children are relatively greater, being 5.6 % at 5 months' gestational age, 4–5 % at birth, and 3 % at 1 year of age [3, 4].

The falciform ligament anteriorly and the lesser omentum and umbilical fissure posteriorly divide the liver into the conventional right and left lobes.

Histological sections through the liver reveal a rather homogeneous landscape of

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hepatocytes periodically infiltrated with vascular tissue and bile ducts. The basic architectural unit of the liver is the liver lobule. The lobule consists of plates of hepatocytes lined by sinusoidal capillaries that radiate toward a central efferent vein. Liver lobules are roughly hexagonal with each of six corners demarcated by the presence of a portal triad of vessels consisting of a portal vein, bile duct, and hepatic artery (Figs. 26.1 and 26.2). Both the portal vein and hepatic artery supply blood to the lobule, which flows through a network of sinusoidal capillaries before leaving the lobule through the central vein. Although hepatocytes are the major parenchymal cell type of the liver and account for 78 % of liver volume [5], they function in concert with cholangiocytes (biliary epithelial cells), endothelial cells, sinusoidal endothelial cells, Kupffer cells (resident liver macrophages), pit cells (natural killer cells), and hepatic stellate cells. Predominant cell types and their functions within adult liver are shown in Table 26.1 [6].

The hepatocytes, which are polarized epithelial cells, are arranged as cords that are one cell thick in mammals. The basolateral surfaces of the hepatocyte face fenestrated sinusoidal endothelial cells, which facilitates the transfer of endocrine secretions from the hepatocytes into the blood stream. Tight junctions formed between neighboring hepatocytes generate a canaliculus that surrounds each hepatocyte and is responsible for collection of bile acids and bile salts that are transported across the hepatocyte's apical surface. Bile collected by the canaliculi is carried to the bile ducts within the portal triad and subsequently transported for storage in the gall bladder. The complex arrangement between the polarized hepatocytes with the capillaries and cholangiocytes underlies both endocrine and exocrine functions of the liver.

Normal Liver Histology

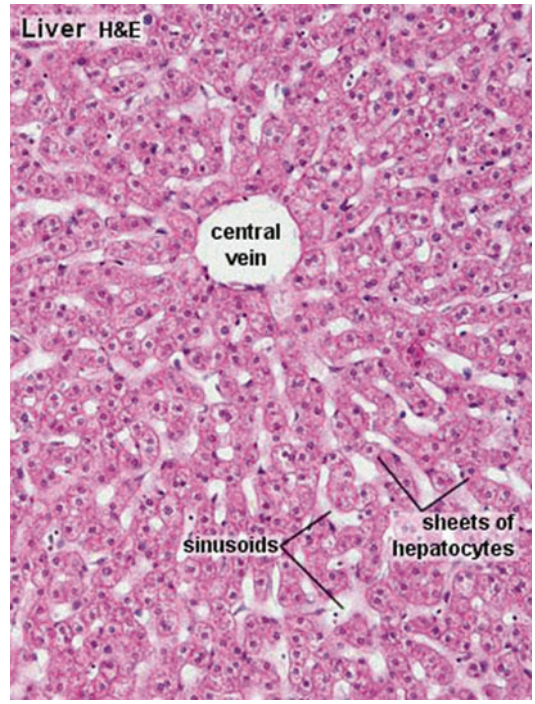

Fig. 26.1 Showing arrangement of hepatocytes in cord around central vein

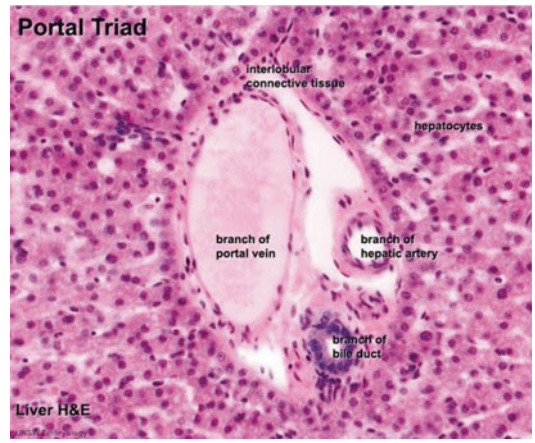

Fig. 26.2 Showing portal traid

Table 26.1 Various cell types and their functions in adult liver [6]

Cell type	Position in liver	Function
Hepatocyte	Parenchyma	70 % of liver cell population
		Protein secretion
		Bile secretion
		Cholesterol metabolism
		Detoxification
		Urea metabolism
		Glucose/glycogen metabolism
		Acute phase response
		Blood clotting
Cholangiocyte/bile duct cell	Duct epithelium	3 % of liver cell population
		Form bile ducts to transport bile
		Control rate of bile flow
		Secrete water and bicarbonate
		Control pH of bile
Endothelial cell	Vasculature	Form veins, arteries, venuoles, and arterioles
		Control blood flow
		_
Liver sinusoidal endothelial cells		2.5 % of lobular parenchyma
		Form sinusoidal plexus to facilitate blood circulation
		Highly specialized
		Allow transfer of molecules and proteins between serum and hepatocytes
		Scavenger of macromolecular waste
		Cytokine secretion
		Antigen presentation
		Blood clotting
Pit cells	Liver natural killer cells	Rare
		Cytotoxic activity
Kupfer cells	Sinusoids	2 % of liver cells
		Scavengers of foreign material
		Secrete cytokines and proteases
Hepatic stellate cells	Perisinusoidal	1.4 % of liver cells
		Maintainence of extracellular matrix, vitamin A, retinoid storage
		Control of microvascular tone
		Activated to become myofibroblast
		Contributes towards regenerative response to injury
		Secretion of cytokine

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Embryologic Liver Morphogenesis

Liver primordium appears in the middle of third week as an outgrowth of endodermal epithelium of foregut (mouse e9). This outgrowth consists of rapidly proliferating cells that penetrate the septum transversum mesenchyme forming, what is called liver bud. As this grows connection between this liver bud and foregut narrows which forms the bile duct. A small ventral out growth from bile duct gives rise to gall bladder and common bile duct.

During further development these form epithelial liver cords which intermingle with vitteline and umbilical veins which forms hepatic sinusoids. These liver cords differentiate in to hepatocytes and biliary epithelial cells, while kupffer cells, connective tissue cells, hematopoietic cells are derived from septum transversum mesenchyme. In the tenth week weight of liver is approximately 10 % of body weight that is due to large no. of sinusoids with hematopoietic progenitors colonizing these sinusoids making it as principal hematopoietic organ (Fig. 26.5). But in last 2 months these hematopoietic progenitor cells decrease in number and liver constitute only 5 % of total body weight [7].

Carnegie stages based on the external and/or internal morphological development of the embryo, developed in Carnegie institute of Washington after studying embryos up to 8 weeks described the liver developmental events according to stages. Summary of key events described are given in Table 26.2

Table 26.2 Summary of key events in liver development according to Carnegie staging (source-UNSW Embryology)

Carnegie stage	Feature
Stage 11 (29 days)	Hepatic diverticulum development
Stage 12 (30 days)	**Cell differentiation**
	Septum transversum forming liver stroma
	Hepatic diverticulum forming hepatic trabeculae
Stage 13 (32 days)	Epithelial cord proliferation enmeshing stromal capillaries
Stage 14 (33 days)	Hepatic gland and its vascular channels enlarge
	Hematopoietic function appeared
Stage 18 (44 days)	Obturation due to epithelial proliferation
	Bile ducts became reorganized (continuity between liver cells and gut)
Stage 18–23 (44–56 days)	Biliary ductules developed in periportal connective tissue
	Produces ductal plates that receive biliary capillaries

Development of Sinusoids and Portal Vein [7]

In the fifth week three major can be distinguished (1) Vitteline veins or omphalomesenteric veins carrying blood from yolk sac to sinus venosus (2) Umbilical veins originating in chorionic villi and carrying oxygenated blood to embryo (3) Cardinal veins draining the embryo proper.

These beds converge on the sinal horns before entering the heart. The left and right vitelline veins are joined by three anastomoses to form a ladder-like structure with the intestinal tract intertwined. The liver cords growing in to septum interrupt the course of veins and extensive vascular network, the hepatic sinusoids. Extrahepatic portal vein develops from these vessels after selective obliteration of portions of the ladder. The left vitelline vein receives a tap from the left umbilical vein. The intrahepatic segment of this tap becomes the umbilical portion of the left portal vein. Flow in this segment reverses after birth and supplies segments of the left hemiliver. As the liver develops, the venous drainage of the parenchyma becomes focused into two vessels, the future right and left hepatic veins, and later the middle vein (not shown), which usually drains into the left hepatic vein. The ductus venosus develops as a through-channel from the left portal vein to the common hepatic vein. The remainder of the portal vein blood perfuses sinusoids before reaching the hepatic veins. The vasculature simplifies with the removal of several segments including the most caudal anastomosis between the vitelline veins, the rostral portions of the left vitelline and left umbilical veins, and the right umbilical vein. The right lobe has grown

faster than the left as the left lobe has lost the supply from the left vitelline vein and left umbilical vein blood is shunted through the ductus venosus. The left umbilical vein actually lies in the midline and later shifts to the right of midline.

Development of the Biliary Tree [8]

For up to 8 weeks of gestation, the extrahepatic biliary tree develops through lengthening of the caudal part of the hepatic diverticulum. The hepatic duct (ductus hepaticus) develops from the cranial part (pars hepatica) of the hepatic diverticulum. The distal portions of the right and left hepatic ducts develop from the extrahepatic ducts and are clearly defined tubular structures by 12 weeks of gestation. The proximal portions of the main hilar ducts derive from the first intrahepatic ductal plates. The extrahepatic bile ducts and the developing intrahepatic biliary tree maintain luminal continuity from the very start of organogenesis throughout further development, contradicting a previous study in the mouse suggesting that the extrahepatic bile duct system develops independently from the intrahepatic biliary tree and that the systems are initially discontinuous but join up later. The normal development of intrahepatic bile ducts requires finely timed and precisely tuned epithelial – mesenchymal interactions, which proceed from the hilum of the liver toward its periphery along the branches of the developing portal vein (Figs. 26.3, 26.4, and 26.5).

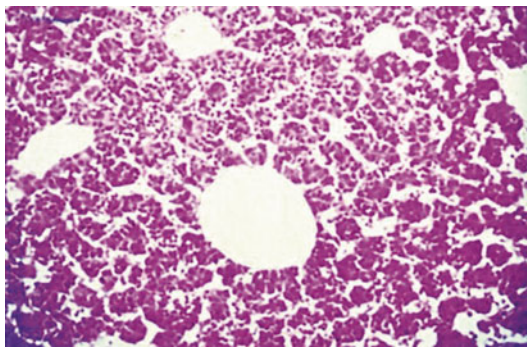

Fig. 26.3 Histological section of liver at 8 weeks of development showing radiating pattern of sinusoids around central vein

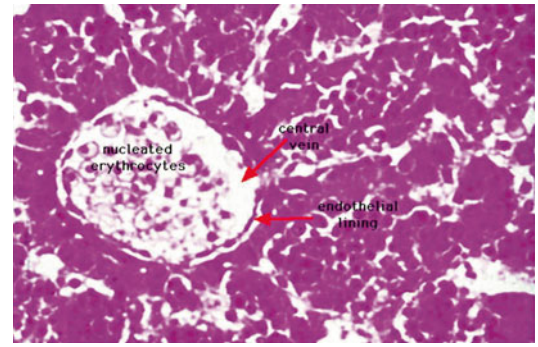

Fig. 26.4 Histological section of liver at 8 weeks showing presence of erythrocytes within central vein and development of endothelial lining

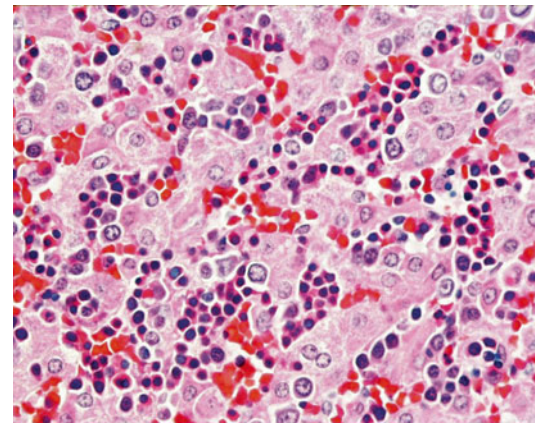

Fig. 26.5 Histological sections of liver at 10 weeks showing hematopoietic cells within sinusoids

Liver Development as Seen in Mouse Embryo

The embryonic liver originates from the ventral foregut endoderm [9], which becomes the hepatic diverticulum, the first morphological sign of the embryonic liver. The hepatic diverticulum is located adjacent to the developing heart and can be identified at E9.0. The anterior portion of the hepatic diverticulum gives rise to the liver and intrahepatic biliary tree, whereas the posterior portion forms the gall bladder and the extrahepatic bile ducts (EHBD). At E9.5, hepatoblasts delaminate from the anterior portion of the

hepatic diverticulum and invade the adjacent septum transversum mesenchyme (STM) to form the liver bud [10]. The STM contributes the fibroblasts and stellate cells of the liver. Between E9.5 and E15, the liver bud undergoes significant growth and, following invasion of hematopoietic cells, becomes the primary site of fetal haematopoiesis.

Hepatoblasts are bi-potential precursor cells which give rise to hepatocytes and biliary epithelial cells (BECs; also known as cholangyocytes), the two major liver cell types. Hepatocytes are parenchymal cells that comprise about 70 % of hepatic cells [11, 12]. BECs line the lumen of the intrahepatic bile ducts (IHBD). The remaining 30 % of the adult liver is comprised of non-parenchymal cells, including Kupffer cells, stromal cells and stellate cells of mesodermal origin.

Triggers for Development

A large no. of transcription factors have been identified which act as signals/triggers for fate specification of cells, differentiation and patterning (regulated assignment of fates over a large area in a manner that ensures proper positional relationship between tissues. This signaling can be cell–cell signaling between neighboring cells or between adjacent cell layers. Such signals can be divided into two classes: *permissive* signals, which allow a tissue to progress to a fate that has already been assigned, and *instructive* or *inductive* signals, which divert a tissue to a new fate that would not otherwise have been followed. Instructive signals play an important role in regulating patterning by committing multipotent cells to specific lineages [13].

Developmental signals have traditionally been identified through transplantation studies, in which different embryonic structures (e.g., epithelium and mesenchyme) are cocultured. The resulting fate (or absence thereof) indicates whether signals are present or absent, and if present, whether the signals are permissive or instructive.

But these signals cannot be considered simply as cause and effect. There occurs reciprocal interaction for example, the epithelium may respond to signal "A" from the mesenchyme by supplying signal "B", which in turn prompts the mesenchyme to secrete signal "C", and so forth. And if one chain of signals is broken, abnormal development ensues, or in other way epithelium may require sequential signals e.g. signal A followed by B and then C.

As development is a highly dynamic process, cells and cell layers are in constant motion relative to each other. Cell or tissue interactions may exist only transiently – long enough for a signal to be received, but not long enough to be easily characterized experimentally. The number of secreted factors encoded in the genome is vast, leading to the potential for a level of signaling complexity that may preclude straight forward analysis.

Sequence of events occurring during liver development and triggers involved can be summarized as

1. **Endodermal fate assignment**

   Definitive endoderm emerges as a sheet of cells from the anterior end of the primitive streak during gastrulation. Signaling by the TGFβ growth factor ***Nodal*** initiates both endoderm and mesoderm formation in a concentration-dependant manner, with low Nodal doses inducing mesoderm and higher doses inducing endoderm [14, 15]
2. **Gut tube patterning and assignment of hepatic fate to endodermal cells**

   Throughout gastrulation and the early somite stages of development morphogenetic movements turn the endoderm into an epithelial gut tube surrounded by mesoderm. During this time the gut tube epithelium is further patterned along the anterior-posterior (A-P) axis into foregut, midgut and hindgut domains by secreted factors from the adjacent mesoderm. These domains can be identified by the expression of transcription factors such as Hhex in the foregut, Pdx1 in the midgut and Cdx in the posterior endoderm [16, 17]. Establishment of the foregut progenitors is important step in hepatogenesis because in vivo only the foregut endoderm (but not the hindgut) is competent to develop into the liver [18–21]. This

intrinsic hepatic potential is probably due to the expression of transcription factors such as Foxa2, Gata4–6 and Hhex, which have important roles in early foregut organogenesis. so these transcription factors give foregut endoderm, anterior endoderm fate.

At early somite stages of development (e8.5; four to seven somites stage in mice), FGF signals from the developing heart and BMPs from the STM induce hepatic fate in the ventral foregut endoderm [18, 22–25]. This has been extensively studied using mouse embryo foregut explants, which when isolated at the two to four-somite stage will express *Albumin* after 1–2 days in culture if the cardiac mesoderm is present. However, if the cardiac mesoderm is removed, or if either FGF or BMP signaling is blocked, liver induction does not occur [22, 23, 26]. Furthermore, exogenous FGF1 or FGF2 can replace the cardiac mesoderm and induce *Albumin* expression in foregut endoderm explants [24]. BMP signaling however is required, but not sufficient, for hepatic induction in explants and may act in part by maintaining *Gata4/6* expression [22]. Mouse explant studies also suggest that different concentrations of FGF are critical for segregating different foregut organ lineages are from a common progenitor cell population, with high, intermediate and low levels of FGF signaling promoting lung, liver and ventral pancreas respectively [27, 28]. It is still unclear whether it is the proximity, or the length of time that the endoderm is in contact with the cardiac mesoderm that controls the FGF dose.

3. **Liver Bud development**

Liver bud development Once hepatic fate is acquired by endodermal cells by signalling from cardiogenic mesoderm, role of homeobox transcription factor Hhex comes in to play which regulates proliferation and positioning of the ventral endoderm within the cardiogenic field that controls induction of hepatic cell fate and is required to ensure pseudostratification. In the absence of Hhex, mutant mouse embryos initiate hepatic specification [29] but fail to complete liver bud morphogenesis, resulting in hepatic structures that lack a parenchymal cell component [30, 31]. Conditional ablation of the Hhex gene in the early hepatoblasts also disrupts their differentiation into hepatocytes [32] suggesting that Hhex has multiple roles in controlling the onset of hepatogenesis. Between the 7 and 11 somite stages of development, in response to the inductive cues from the heart and mesenchyme, the cells forming the hepatic endoderm that lie proximal to the sinus venosus transition to a columnar morphology [33] and express several hepatic genes including Albumin, Afp, Ttr (transthyretin), Rbp (retinol binding protein), and the transcription factor Hnf4a, all of which are reliable indicators of early hepatic cell fate

4. **Liver bud growth and migration of cells in to septum transversum mesenchyme**

At around 21 somites in the mouse, nuclear migration within the epithelial cells results in a pseudostratified epithelial morphology. The transition in cellular morphology results in a thickening of the epithelium, which bulges into the surrounding stroma. The basal face of the diverticulum is surrounded by a matrix which contains laminin, nidogen, type IV collagen, fibronectin, and heparin sulfate proteoglycans [34]. The matrix surrounding the basal surface of the epithelium is then degraded and E-cadherin expression is downregulated in the hepatic cells as they delaminate and invade the surrounding stroma as migrating cords of hepatoblasts [35]. The migration of the hepatic progenitor cells into the stroma requires the action of matrix metalloproteinases (MMPs) [36]. Several MMPs have been identified in the vicinity of the liver bud including MMP-14 in the hepatic progenitors and MMP-2 in the surrounding mesenchyme. A number of other transcriptional regulators have been characterized as playing a role in later events For example, the homeodomain factors HNF6 (also called Onecut-1) and Onecut-2 are required for hepatoblast migration [37]. Homeobox transcription factor Prox1 also promotes hepatoblast proliferation and migration from the primary liver bud

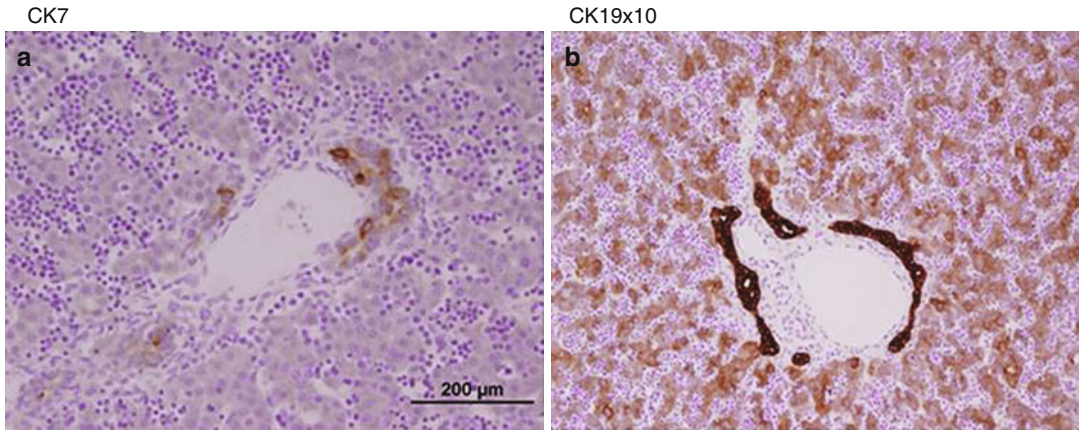

Fig. 26.6 (**a**, **b**) Histological sections of liver at 10 week with immune staining for cytokeratin 19 and cytokeratin 7 marking the differentiation of biliary epithelial cells

[38]. Although the mechanism through which Prox1 controls hepatoblast migration is unclear, the mutant hepatoblasts were found to maintain high levels of E-cadherin and failed to degrade the basal matrix surrounding the liver bud.

In *Prox1*-/- embryos hepatoblasts are specified and begin to proliferate, but the basal lamina fails to degrade and the cells remain trapped in the hepatic diverticulum. OC-1 and OC-2 are expressed in the foregut endoderm and hepatoblasts, and are redundantly required for the timely degradation of the basal lamina [37]. At e9.5, *OC-1*;*OC-2* double mutants resemble the *Prox1*-/- phenotype, but later hepatoblast invasion recovers resulting in a hypoplastic fetal liver. These mutant livers also have bile duct and gall bladder defects due to later roles of OC-1 and OC-2

The importance of cell-ECM interactions is also illustrated by the fact that hepatoblasts deficient for the laminin receptor β1-integrin are unable to colonize the liver bud At e9.0, prior to vascularization of the liver bud, endothelial precursor cells lay between the hepatic epithelium and the STM. This close contact with blood vessels persists as hepatoblasts migrate into the stroma. Null mutations in the vascular endothelial growth factor receptor gene *Vegfr-2* results in embryos that lack endothelial cells and the hepatoblasts fail to delaminate in these embryos [39]. In addition angiogenesis inhibitors repress liver bud growth in culture, suggesting that endothelial cells provide unknown paracrine factors promoting hepatoblast migration and/or proliferation

5. **Differentiation of hepatocytes and biliary epithelial cells**

The hepatoblasts that migrate into the septum transversum appear to have the potential to differentiate into either cholangiocytes or hepatocytes. As evidenced by expression of genes associated with adult hepatocytes (hnf4alpha, albumin) and biliary epithelial cells (cytokeratin 19) as well as fetal liver genes such as AFP. Hepatoblasts in contact with the portal veinform a monolayer, and then a bi-layer, of cuboidal biliary precursors that increase cytokeratin-19 (CK-19) expression and down-regulate hepatic genes (Fig. 26.6). Between e17 and into the perinatal period focal dilations appear in the bilayer and these become surrounded by portal mesenchyme to form IHBD, while the remaining bi-layer cells regress. This process, which involves tubulogenesis and apoptosis, is known as ductal plate remodeling. Hepatoblasts in the liver parenchyma that are not in contact with portal veins gradually differentiate into mature hepatocytes. At e17 hepatocytes acquire their characteristic epithelial morphology arranged in hepatic

chords with bile canaliculi on the apical surfaces

6. **Maturation of the hepatocytes**.

As stated earlier The hepatoblasts that migrate into the septum transversum have the potential to differentiate into either cholangiocytes or hepatocytes. Cells that follow a hepatocyte cell fate progressively mature and progressively accumulate the gene expression and physiological profile of mature hepatic parenchyma cells [40, 41]. The maturation of hepatocytes is facilitated through network of transcription factors that regulate hepatocyte gene expression. By comparing developmental time points, the complexity of cross-regulation among factors is increasing gradually as development is progressing and the more number of transcription factors binding a given promoter are being identified. various transcriptional factors interact to form a circuit which ensures terminal differentiation of hepatocytes as development progresses. Six transcription factors, (HNF1a, HNF1b, FoxA2, HNF4a1, HNF6, and LRH-1 [Nr5a2]), have been found to form the core of this regulatory circuitry by occupying each other's promoters as well as the promoters of peripheral hepatic transcription factors., gene deletion studies in mouse embryos have found that HNF1b, FoxA2, and HNF6 all have roles in controlling the onset of hepatic gene expression during specification and liver bud formation, which is consistent with these factors having important roles in establishing the transcription factor network within the liver progenitor cells. HNF4a is required for development of normal architecture development. When HNF4a is specifically removed from fetal hepatoblasts, hepatic architecture is severely affected, with livers exhibiting loss of endothelial cells and disrupted hepatocellular polarity. The loss of hepatocyte polarity in Hnf4a-/- livers appears to reflect a requirement for HNF4a in controlling expression of several proteins involved in cell junction assembly [42]. Moreover, in the absence of HNF4a the core regulatory network is severely disrupted in fetal hepatic progenitors [43]; however, in adult hepatocytes, maintenance of the transcription factor network appears to be less dependent on HNF4a, although HNF4a does continue to have an important role in maintaining adult hepatocyte function [44].

Another important aspect in hepatocyte maturation is generation of membrane polarity Which directs molecules to sinusoidal or basolateral membrane. In non polarized hepatocytes progenitor cells, would be sinusoidal and canalicular membrane proteins are sorted at transgolgi network in to distinct transport vesicles by basolateral sorting signals and raft mechanisms.

Polarity is established as the liver bud invades the periheaptic mesoderm and likely mechanism as suggested by Yeaman et al. is as a result of from cell- cell, cell mesenchymal interaction which establishes membrane asymmetry triggering the events of polarization which includes establishment of apical junctional complexes (tight junction and cadherin adherens junction) and restriction of signaling events and polarized attachment of actin and microtubules, which then lead to the reorganization of the cytoskeleton in the cell [45].

7. **Developmental interaction of hepatocytes and hematopoietic progenitor cells**

Liver is primary hematopoietic organ during fetal development because in mammals soon after endodermal cells with hepatocytic fate enters the surrounding mesenchyme hematopoietic progenitors colonize the developing liver and this is because liver progenitor cells generate an environment that support hematopoiesis as suggested by coculture studies [46] and as the hepatocytes mature and undergo further differentiation they loose the property to support hematopoiesis resulting in change of hematopoiesis site from fetal liver to bone marrow.

This interaction between hematopoietic cells and hepatocyte progenitors is not one way because these hematopoietic cells secrete cytokine oncostatin M which in conjunction with other factors e.g. HGF and wnt promote hepatocyte maturation [47–51]. OSM induces metabolic maturation by activating the gp130

receptor and a JAK/Stat3 signaling pathway [52, 53], while promoting morphological maturation into polarized epithelium via K-ras and E-cadherin [48, 54]. Some evidence suggests that HGF and OSM activity is balanced by TNFα, which inhibits maturation and maintains the proliferative capacity of fetal hepatocytes, allowing the liver to grow to the appropriate size before differentiating [55]

8. **Origin of hepatic stellate cells and kupffer cells**

   The origin of hepatic stellate cells has been debated, with various lines of evidence suggesting that the cells are of endodermal, neural crest, or mesenchymal origin. Most of the conclusions drawn from such studies were based on expression of shared sets of marker genes; however, shared gene expression does not necessarily correlate with cell lineage. Direct lineage tracing experiments have been performed in avian embryos [56], which led to the conclusion that mesothelial cells derived from the proepicardium and septum transversum mesenchyme could give rise to both endothelial and stellate cells within the hepatic sinusoids. Recent studies in human [57] and in mouse [58] support a mesothelial origin of hepatic stellate cells.

9. **Development of biliary tree**

   The extrahepatic biliary tract originates from a portion of the ventral endoderm that is positioned immediately rostral to the ventral pancreatic bud. Reports demonstrate that the extrahepatic biliary tract derives from pancreatobiliary precursors coexpressing PDX1 and SOX17 [59]. This precursor population gives rise to SOX17+/PDX1-extrahepatic biliary cells and SOX17-/PDX1+ pancreatic cells. The segregation of the pancreatobiliary precursor population depends on SOX17. This factor is required for extrahepatic biliary tract development and overexpression inhibits pancreas development. The expression of SOX17 is controlled by homolog of hairy/enhancer-of-split (Hes-1): in the absence of Hes-1 the mice not only display accelerated differentiation of pancreatic endocrine cells from pancreatic progenitors [60], the bile duct cells also differentiate to a pancreatic phenotype [61, 62]. Other transcription factors involved in extrahepatic biliary development include Hhex: in Hhex null embryos the common bile duct is replaced by duodenal-like tissue suggesting that the decision between a dudodenal or biliary fate appears to depend, at least in part, on the function of this transcription factor. Mice deficient in HNF6, Hes-1, HNF1b, or FoxF1 show lack or abnormal shape of the gallbladder [63–65].

   Intrahepatic biliary tree develops as ductal plate which is continuous ring of cells arranges as monolayer that surrounds periportal mesenchyme. TGFβ, Wnt and Notch are candidate signals from periportal mesenchyme that promotes BEC development. There is evidence that a TGFβ signaling gradient emanating from the portal region promotes biliary differentiation in the adjacent hepatoblasts [66–68]. Wnt/β-catenin signaling also promotes BEC development [69–71], and may act in part by stimulating the expression of EGF, which along with HGF can induce the formation of biliary structures in cultured hepatocytes [49, 72, 73].

   The Notch signaling pathway is also considered contributing to biliary development based on the finding that patients affected with Alagille syndrome, a disease with bile duct paucity, had mutations in the JAGGED1 and NOTCH2 genes [74–76]. The analysis of this pathway in the liver has been challenging due to the presence of multiple ligands and receptors with overlapping functions. However, recent data favor a model in which Notch signaling controls multiple steps in biliary development, including the initial differentiation of cholangiocytes. Analysis of mice that have a liver-specific inactivation of RBP-Jk, a common transcriptional mediator of Notch signaling, revealed a reduced number of biliary cells differentiating from hepatoblasts [77]. Since expression of Jagged1 occurs in the periportal mesenchyme and biliary cells while Notch2 is present in the biliary cells [78], it appears that Notch signaling contributes not only to differentiation of biliary cells but also restricts differentiation to a periportal location.

Liver Stem Cells

The liver has remarkable regenerative capacity and can regrow when up to 70 % of its mass is removed. Liver regenerates primarily through proliferation of mature hepatocytes [79]. However adult liver also contains hepatic progenitor cells. Adult hepatic progenitor cells are believed to provide a backup system for replenishing hepatocytes and biliary epithelial cells when the regenerative capabilities of these cells are impaired such as in chronic injury states. These progenitor cells emerge and expand in periportal areas of the injured mouse, rat and human liver. Hepatic progenitor cells reside within or derive from the epithelial lining of bile ducts has been confirmed in mice by lineage tracing of cells expressing the transcription factor Sox9 [80]. This reserve compartment of hepatic progenitor cells is activated when mature hepatocytes and cholangiocytes are damaged or inhibited in their replication, The activation of the stem cell compartment, referred to as a "ductular reaction" in humans and "oval cell reaction" in rodents, is observed in circumstances of prolonged necrosis, cirrhosis, and chronic inflammatory liver diseases. This process involves expansion of bipotential transit amplifying progenitor cells, which can differentiate into hepatocytes and biliary cells. Intermediate hepatocytes, with an intermediate phenotype between progenitor cells and mature hepatocytes, are seen in moderate to severe inflammatory hepatitis [81].

Liver Stem Cells and Disease States/ Hepatocellular Carcinoma

The existence of a hepatic stem cell compartment gave rise to expectations regarding the practical applications of such research. Understanding and identification of the bipotential progenitor cells may hold promise for new therapeutic treatments to a wide range of liver pathological conditions ranging from congenital metabolic diseases, end-stage liver cirrhosis, and hepatocarcinogenesis. Existence of cancer stem cells (CSCs) was first proposed over 40 years ago, but have these cells been identified in past decade, in hematological malignancies, and more recently in solid tumors that include liver, breast, prostate, brain, and colon. Constant proliferation of stem cells is a vital component in liver tissues.

Exploration of the difference between CSCs from normal stem cells is crucial not only for the understanding of tumor biology but also for the development of specific therapies that effectively target these cells in patients. These ideas have drawn attention to control of stem cell proliferation by the transforming growth factor beta (TGF-β), Notch, Wnt, and Hedgehog pathways. Recent evidence also suggests a key role for the TGF-β signaling pathway in both hepatocellular cancer suppression and endoderm formation, suggesting a dual role for this pathway in tumor suppression as well as progression of differentiation from a stem or progenitor stage. So understanding the mechanism of development and differentiation of stem cells will help us target CSCs via inducing differentiation of CSCs into nontumorigenic cells or completely eliminating the cells via inhibition of the self-renewing stem-cell state [81].

Developmental Anomalies of Liver

Variation in lobulation and vascularity are the most common developmental anomalies (or variations) during development but these are not clinically significant. But their knowledge becomes important in pathological conditions as in surgeries for GB or other surrounding organs.

One classical example of abnormal development is Alagille syndrome (ALGS) which is a complex autosomal dominant disorder due to defects in the Notch signaling pathway. Main clinical features and malformations are chronic cholestasis due to paucity of intrahepatic bile ducts, congenital heart disease primarily affecting the pulmonary outflow tract and vasculature, butterfly vertebrae, characteristic facies with a broad forehead, posterior embryotoxon and/or anterior segment abnormalities of the eyes, and pigmentary retinopathy. The importance of Notch signaling and why it results in abnormal biliary development has been discussed earlier in development of biliary tree.

Implications

Understanding the mechanism of development and differentiation of hepatocytes and applying these developmental protocols on mouse and human embryonic stem cells, researchers have successfully generated cultures where up to 70 % of the cells exhibit a "hepatocyte-like" phenotype. These cells exhibit many features of hepatocytes including; (1) expression of hepatic enzymes, (2) hepatocyte morphology, (3) robust glycogen storage, (4) uptake and metabolizism of drugs and (5) secretion of albumin [82].

These hepatocytes can be used as

- Tool to rescue liver function in disease states
  Several groups have found that transfusion of embryonic stem cells derived hepatocytes into mice with various liver injury models exhibited a modest rescue of liver function, although engraftment of the cells into the host liver was very low [82]. So one of the concerns about transplanting cells into a liver undergoing failure is that the environment of the liver may not allow the graft to 'take'.
- Artificial liver devices
  There is possibility of creating an artificial liver, similar to a dialysis machine for kidney failure, that would perform all the functions of a normal liver which might replace the need for transplants or provide a bridge to therapy for patients waiting for transplants. Despite providing some respite for patients, these artificial liver bioreactors have not provided any overall survival benefit or reduced the need for transplants. In addition, obtaining huge numbers (10^{10}) of fresh hepatocytes to load into the bioreactors is an ongoing issue.
- Neo Livers [83]
  Researchers at the McGowan Institute for Regenerative Medicine in Pittsburg are trying alternate approach where they co-opt part of the body, in this case the lymph node, to act as a reservoir for transplanted hepatocytes. The hepatocytes grow well in the lymph nodes, perhaps because there is a lot of blood supply to the nodes, and the resultant 'hepatasized lymph nodes' are even able to rescue mice with lethal liver failure. One issue with translating this approach to humans is the risk of hepatocyte rejection given the donor population of hepatocytes. Researchers theorize that someday perhaps induced pluripotent cells created from the patients themselves could generate the hepatocytes needed for the transplant and thus circumvent the issue of graft rejection.
  In an international collaboration, researchers from the USA, Italy and Japan are attempting to create '**neolivers**' by reseeding livers stripped down to a three dimensional scaffold with fresh hepatocytes. They have had some success testing this approach in rats and are now optimizing their seeding strategies and making sure that the stripping technique does not compromise the ability of the neoliver to be infiltrated with blood vessels.

Drug Testing

Pharmaceutical companies require large numbers of hepatocytes in order to identify which chemicals could be potential new drugs. This procedure is invaluable because 50 % of drugs are taken off the market because of toxicity to the liver. Currently, hepatocytes left over from transplants or hepatocyte cell lines derived from liver cancers are used as sources for drug testing but variation and functionality are issues that can confound the results. This is where stem cells could be a tremendous boon. Stem cells could theoretically generate limitless numbers of hepatocytes, and induced pluripotent stem cells could even provide patient-specific hepatocytes to verify that a drug therapy would not harm the patient's liver [83].

Conclusion

Through rapid evolution of molecular genetics and advancements in field of experimental developmental biology with development of new animal models our understanding of molecular mechanisms controlling liver development is improving. These findings of molecular mechanisms are applicable to all tissues and organs. In last decade researchers have uncovered many genes regulating hepatogenesis and transcription factors involved but as the knowledge is improving more questions are

also coming up after observations such as why hepatocytes when placed in culture system dedifferentiate, what regulates organ size. As our understanding is improving it is likely that with time we will get answers to many questions and many answers to the questions known till date are likely to change.

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Part VIII

Fetal Cardiovascular Development: Up to Second Trimester

Embryological Basis for Vascular Anomalies

27

Trenton R. Foster, Jason A. Chin, Kirstyn E. Brownson, Hualong Bai, and Alan Dardik

Formation of the mature vascular system occurs through a well organized process beginning early in the embryo. Deviation from the normal sequence of events leads to well described vascular anomalies. The cause for deviations from normal development often remains unclear, although research has identified certain signaling pathways that are essential to normal development. This chapter describes an embryologic basis for a variety of vascular anomalies.

Vasculogenesis

Vasculogenesis is the process by which a new vascular system is created de novo in the early embryo, e.g. the formation of endothelial cells and tubes from previously undifferentiated cells. This process is directed by molecular signaling pathways beginning during the third week in utero. At this early stage of development, the yolk sac's mesoderm forms hemangioblastic aggregates that contain a core of hematopoietic stem cells and an outer layer of endothelial cells. This aggregation is guided by the influence of signaling molecules including Indian hedgehog, bone morphogenic protein, and transforming growth factor β (TGF-β). The hemangioblastic aggregates coalesce to form the connecting stalk and the chorion. In a similar fashion, the embryonic endoderm secretes bone morphogenic protein and TGF- β to induce vessel formation from the embryonic splanchnic mesoderm. The embryonic splanchnic mesoderm develops a cardiogenic area by creating a horseshoe shaped pair of endocardial tubes that will ultimately give rise to the heart. The splanchnic mesoderm also forms paired dorsal aortas that connect to the endocardial tubes.

After vasculogenesis forms the early vessels, the primitive vascular elements develop buds and sprout new branches. The process of developing new branches from existing vascular structures is termed angiogenesis, and the dilation of these vessels into yet larger ones is termed arteriogenesis. The branches develop into capillary networks that remodel into arterial, venous, or lymphatics, under the influence of local signaling molecules. A diagram of the developing embryonic vascular system is shown in Fig. 27.1. Vasculogenesis is typically restricted to embryogenesis and not normally thought to occur in adults. However, angiogenesis and arteriogenesis occur in adults; an example in a patient with peripheral arterial disease is shown in Fig. 27.2.

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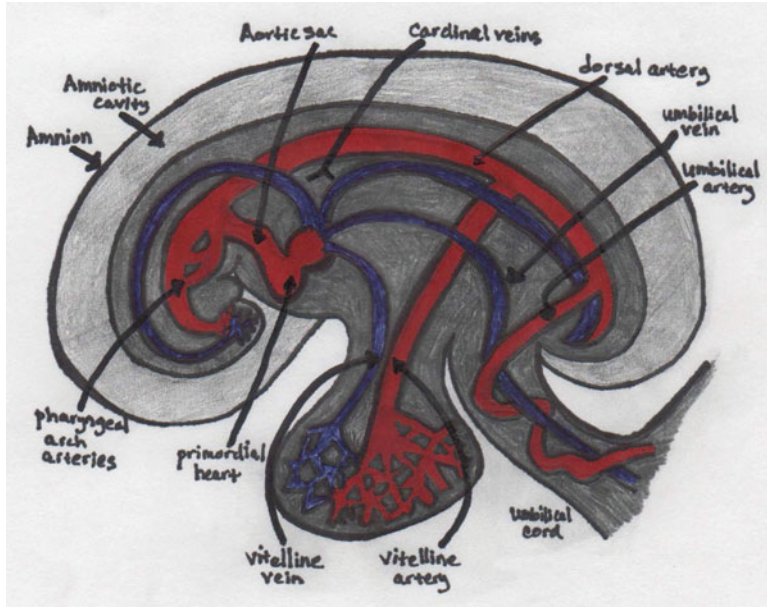

Fig. 27.1 Normal formation of the vascular system in utero showing the primitive arterial and venous network

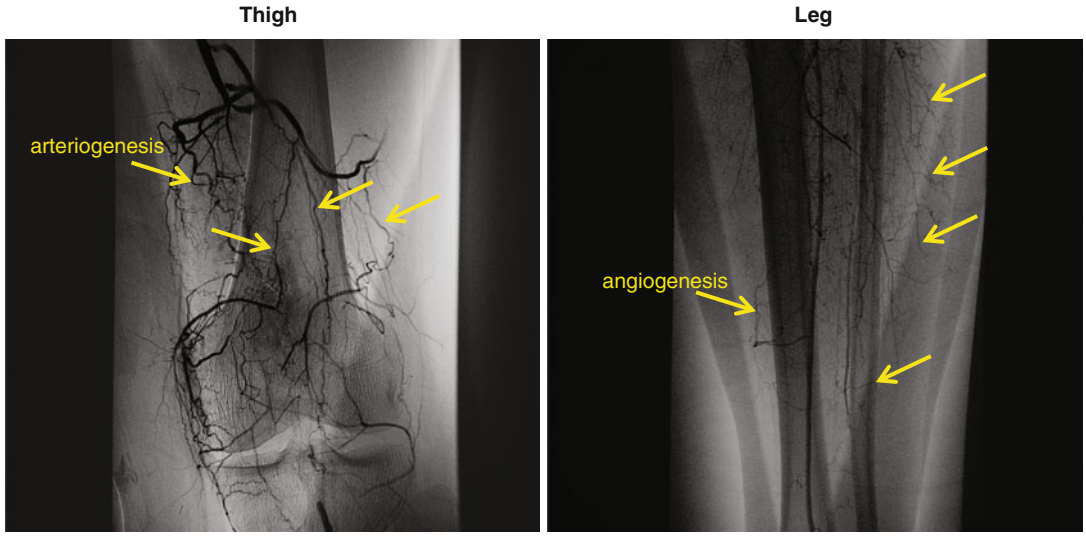

Fig. 27.2 Arterial angiography of lower extremity with arterial occlusive disease. *Left panel* shows the distal thigh including the area of popliteal artery occlusion, as well as collateral circulation around the occlusion (*yellow arrows* show arteriogenesis). *Right panel* shows the leg of the same patient, with capillary growth in the distal muscle (*yellow arrows* show angiogenesis)

VEGF is an important growth factor that promotes the development of fenestrated endothelium found in capillaries, endocrine organs, and the kidneys. Angiopoietin induces the formation of vascular structures containing tight junctions which are essential in the central nervous system to maintain a blood brain barrier. Angiopoeitin-2 and Nitric Oxide synthase are critical for the development of vascular structures. Hypoxia is also known to induce angiogenesis through upregulation of angiogenic genes [1].

Vascular Identity

There are distinct differences between arterial and venous cells. The identity of cells is genetically determined and conferred through signaling pathways. VEGF is common to the development of both arterial and venous cells although differences in the receptor complexes have been identified. Arterial cells are generated from the binding of VEGF to VEGF receptor 2 (VEGFR2) associated with the co-receptor neuropilin-1 (NP1). The VEGFR2-NP-1 complex initiates a signaling cascade that stimulates the delta-notch pathway resulting in the expression of Ephrin-B2, an arterial specific cell surface marker. Venous identity develops when the transcription factor COUP-TFII suppresses the delta-notch pathway, allowing constitutive expression of Eph-B4, a venous specific cell surface marker. Eph-B4 and Ephrin-B2 are transmembrane tyrosine kinase molecules that are critical in vascular development and remodeling [2]. Figure 27.3 shows VEGF signaling with differentiation toward arterial or venous fated cells. Figure 27.4 shows staining of a full-term human umbilical cord comparing arterial and venous endothelial markers.

Lymphatics are created as buds off the developing venous system. The differentiation of lymphatic endothelium from veins occurs under the stimulation of Prospero-related homeobox- 1 (PROX1) gene [3].

Aortic Arch Development

The developing embryo contains two endocardial tubes that fold and join along the midline to create the primitive cardiac area. The cardiac area contains an inflow end and an outflow end. Approximately at days 22–24, the heart moves into a more ventral position causing a bend to develop along the outflow tract forming the first aortic arch. There are a total of six paired aortic arches that will develop from corresponding pharyngeal arches in a rostral to caudal direction. Aortic arches 1 and 2 quickly regress in normal human development, contributing little to adult structures. The third aortic arch becomes the common carotid arteries and proximal internal carotid arteries. The dorsal aortas regress between arches 3 and 4 causing blood to flow selectively through the third aortic arch destined for the head and neck.

The left fourth aortic arch forms part of the arch between the left common carotid and left subclavian arteries. The right fourth aortic arch forms the proximal right subclavian artery.

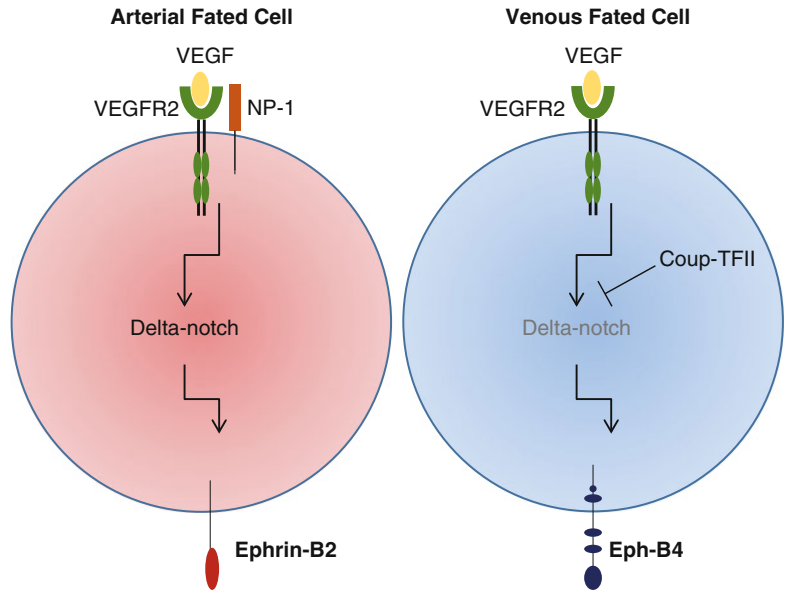

Fig. 27.3 Arterial fated cell expresses Ephrin-B2 via signaling of the delta-notch pathway. Venous fated cell expresses Eph-B4 due to suppression of this pathway by COUP-TFII

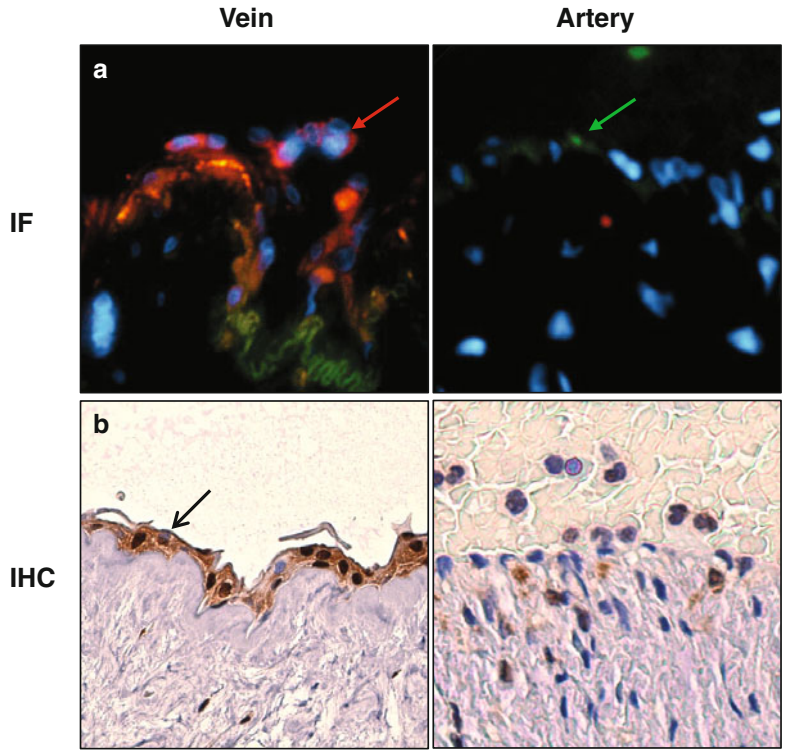

Fig. 27.4 Photomicrographs of a human umbilical cord examined immediately after delivery. (**a**) *Upper row* uses immunofluorescence to show venous endothelium expressing Eph-B4 (*left panel, red arrow*) and arterial endothelium expressing Ephrin-B2 (*right panel, green arrow*). (**b**) *Lower row* uses immunohistochemistry to show venous and arterial endothelium stained for Eph-B4; positive Eph-B4 staining is identified only in the venous endothelium (*left panel*), as marked with *black arrow*, but not in the arterial endothelium (*right panel*)

Additional portions of the right subclavian artery are derived from the right dorsal aorta. The fifth aortic arch does not develop in humans. The sixth aortic arch connects the left pulmonary artery to the left dorsal aorta forming the ductus arteriorsus.

Patent Ductus Arteriosus

The most common vascular anomaly is a patent ductus arteriosus. This connection is essential in fetal life as a mechanism to shunt blood away from the immature lungs to the aorta. Premature closure of the ductus arteriosus results in fetal hydrops. Patency of the ductus arteriosus is maintained by molecular signals including prostaglandin, prostacyclin, and low oxygen concentration. After birth, smooth muscle cells surrounding the ductus are exposed to increased oxygen and a fall in prostaglandin and prostacylcin concentration. These changes induce constriction of smooth muscle cells to close the ductus arteriosus, leaving a remnant structure termed the ligamentum arteriosus. Failure of the ductus to close after birth results in a patent ductus arteriosus (PDA). PDA is known to occur in at least 1/2000 term births with some estimates indicating incidence as high as 1/500. There is a genetic predisposition to PDA although the exact mechanism remains unclear. PDA has also been associated with in utero infections including rubella and particular environmental exposures. The long term consequence of PDA is pulmonary hypertension and eventual heart failure. Management of PDA is guided by the severity of left to right shunting. When indicated, closure is performed by transcatheter or surgical approaches [4].

Coarctation of the Aorta

A similar signaling process that induces closure of the ductus arteriosus is thought to contribute to coarctation of the aorta. Aortic coarctation occurs in the 1/2500 births. The exact mechanism of coarctation is unclear and is likely to have a multifactorial etiology. However, it is thought that

changes in flow dynamics and signaling molecules induce constriction of muscle cells in the aortic wall in the region near or immediately distal to the ductus. Over time, the aorta remodels due to this constriction resulting in the formation of collateral vessels with increased flow around the coarctation through the intercostal arteries. The increased flow through intercostals results in notched ribs, a hallmark of this condition. Long term consequences of coarctation include hypertension and eventual heart failure [5].

Anomalous Aortic Arch

The transition from paired aortic arches to only a left sided aortic arch requires a particular sequence of development and regression. The arch originates as paired aortic arches arising from the cardiac outflow that bend caudally and fuse distal to the seventh intersegmental artery. The fused dorsal aorta continues distally through the developing embryo. In normal development, the right dorsal aorta forms the proximal right subclavian artery. Distal to the right subclavian artery, the right dorsal aortic arch normally regresses and the connection from the right arch to the aorta is thus lost. If regression of the distal right dorsal aorta fails to occur or if there is abnormal regression of the left side, then an anomalous aortic arch is created. Anomalies of the aortic arch may create vascular rings around the trachea or esophagus which can cause compression of these structures.

Specific examples of aortic arch anomalies include a double aortic arch resulting from failed regression the right aortic arch distal to the right subclavian artery. This failed regression forms a double aortic arch as diagramed in Fig. 27.5.

The segment of abnormally retained right dorsal aorta generally traverses posterior to the esophagus before joining the left aortic arch which passes anterior to the trachea. This configuration results in a vascular ring. A vascular ring can also occur from development of a right aortic arch instead of a normal left sided arch. In this case, the right aortic arch is the conduit to the distal aorta while the left aortic arch regresses.

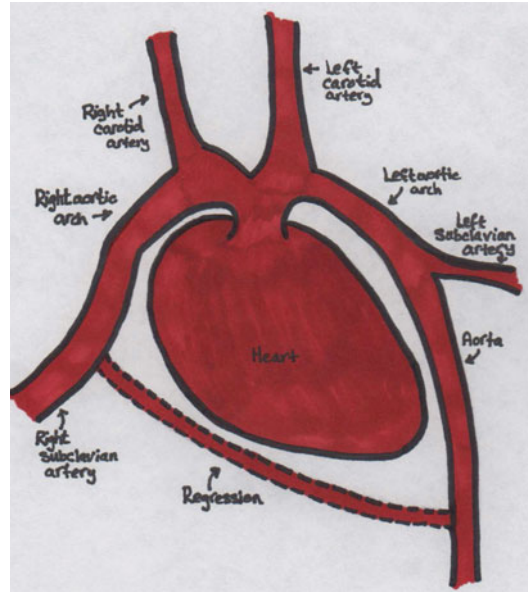

Fig. 27.5 The right aortic arch undergoes selective regression of the segment distal to the right subclavian artery, leaving only a normal left sided aortic arch

The right aortic arch gives rise to the ligamentum arteriosum which connects to the aorta by crossing anterior to the esophagus. If the right aortic arch crosses over to the left side behind the esophagus, then a vascular ring around the esophagus is created with the ligamentum arteriosum. If the aortic arch crosses over anterior to the esophagus and trachea, no vascular ring is formed.

Anomalous Subclavian Artery

The right subclavian artery normally arises from the right aortic arch. Inappropriate involution of the right fourth aortic arch causes aberrant development of the right subclavian artery originating from the left side. The aberrant right subclavian artery crosses from the left sided aorta to the right upper extremity. This path generally occurs behind the esophagus but it can occur between the esophagus and the trachea or in front of the trachea. The aberrant course of the subclavian artery can cause compressive symptoms as it passes around the esophagus and trachea, termed

dysphagia lusoria. Aberrant subclavian artery is also associated with development of an aneurysm at its origin, known as Kommerell's diverticulum, which can also cause compressive symptoms or rupture. However, the majority of patients with aberrant subclavian arteries are asymptomatic [6].

Persistent Median Artery and High Radial Artery

As development continues, segments are created along the length of the embryo, in a body plan common from fruit flies to humans. Each segment is supplied by intersegmental vascular branches. These intersegmental branches are found throughout the cervical, thoracic, and lumbar regions. Development of the arterial supply to the upper extremities originates from the seventh cervical intersegmental artery that forms the axillary artery. The axillary artery continues to the brachial artery that connects to the hand through the median artery. A series of branching and regression then occurs to form the final configuration of the ulnar and radial arteries. During this process, the median artery regresses. Failure of regression results in persistence of the median artery, occurring in 12 % of the population.

During normal development, the ulnar artery originates from the brachial artery and supplies the superficial palmar arch. The radial artery originates from the proximal brachial artery and supplies the deep palmar arch. A final division occurs in which a new, more distal connection develops from the brachial artery to the radial artery. The original connection from the radial artery to the proximal brachial artery then regresses. Failure of this final revision results in a preserved connection of the radial artery to the proximal brachial artery, the basis for a high radial artery [7].

Renal Artery Variations

In the thoracic region, intersegmental branches form intercostal arteries. In the lumbar region, dorsal branches become lumbar arteries and the common iliac arteries. The lumbar ventral branches form allantoic vessels that become umbilical arteries while other paired branches will form the vitelline arteries. The vitelline arteries fuse to form the three dominant vessels to the gut, the celiac artery, the superior mesenteric artery, and the inferior mesenteric artery. Lateral segmental branches also supply the developing kidneys that are migrating rostrally. During the migration, a series of arterial connections are created and then regress. The process of forming arterial connections and regression during renal migration is prone to develop variations in final renal artery configuration. These common variations are important to recognize when performing abdominal operations involving the renal vasculature.

Persistent Sciatic Artery

The lower limb arterial supply is normally via the external iliac artery through the iliofemoral vessels. There is a contribution from the internal iliac artery through an axial artery although this usually regresses with only remnants persisting as the sciatic artery, part of the popliteal artery, and part of the peroneal artery. However, if the iliofemoral artery fails to develop properly, the sciatic artery may persist as the dominant vessel. In cases of complete sciatic preservation, blood travels from the internal iliac through the sciatic and into the popliteal artery to bypass an undeveloped or underdeveloped iliofemoral vessel. There are also 11 cases of persistent sciatic artery in combination with a normal ileofemoral vessel. The etiology of these anomalies is unknown. The location of the abnormal persistent sciatic artery is typically superficial to the buttocks leaving it susceptible to trauma. The majority of patients with a persistent sciatic artery develop symptoms of lower extremity claudication, ischemia, or neurologic sequelae. The neurologic symptoms are often due to compression of the sciatic nerve. Aneurysm of the sciatic artery is also described [8].

Popliteal Entrapment

Popliteal entrapment can occur due to abnormal development of the popliteal fossa. During embryologic development, there is medial

migration of the medial head of the gastrocnemius muscle attachment. If this migration is incomplete or delayed, the abnormal lateral attachment of the gastrocnemius muscle displaces the popliteal artery. As the muscle contracts, it compresses the popliteal artery resulting in an anatomic popliteal entrapment syndrome. Anatomic popliteal entrapment is diagramed in Fig. 27.6.

Functional popliteal entrapment is also described. In cases of functional entrapment, the popliteal fossa is normally developed yet patients demonstrate compression with particular movements of the foot. Hypertrophied gastrocnemius muscle from repetitive use was previously believed to be the cause of functional entrapment; however, this assertion remains controversial as there are young untrained people also demonstrating popliteal artery compression [9].

Venous Anomalies

Superior Vena Cava

The superior vena cava normally develops from the right anterior cardinal and right common cardinal veins. The left common cardinal vein becomes the coronary sinus. Cranially, the internal jugular veins develop from the paired anterior cardinal veins. The internal jugular veins join with the external jugular veins draining the face. The subclavian veins arise from the upper limbs and also connect to drain into the anterior cardinal veins. The left and right anterior cardinal veins connect to form the left brachiocephalic vein with regression of the left anterior cardinal vein caudal to this point. Regression of the left side results in a normal right sided superior vena cava. In the case of the opposite situation, where the right anterior cardinal vein regresses instead, an anomalous left sided superior vena cava is created. A double superior vena cava develops when there is no regression of the anterior cardinal vein on either side leaving both anterior cardinal veins patent.

Inferior Vena Cava

The inferior vena cava develops from a series of connections and regression originating from the posterior cardinal veins. Subcardinal veins develop with connections to the posterior cardinal veins. The subcardinal veins become dominant and both of the posterior cardinal veins regress. The subcardinal veins fuse to form the suprarenal inferior vena cava. The infrarenal

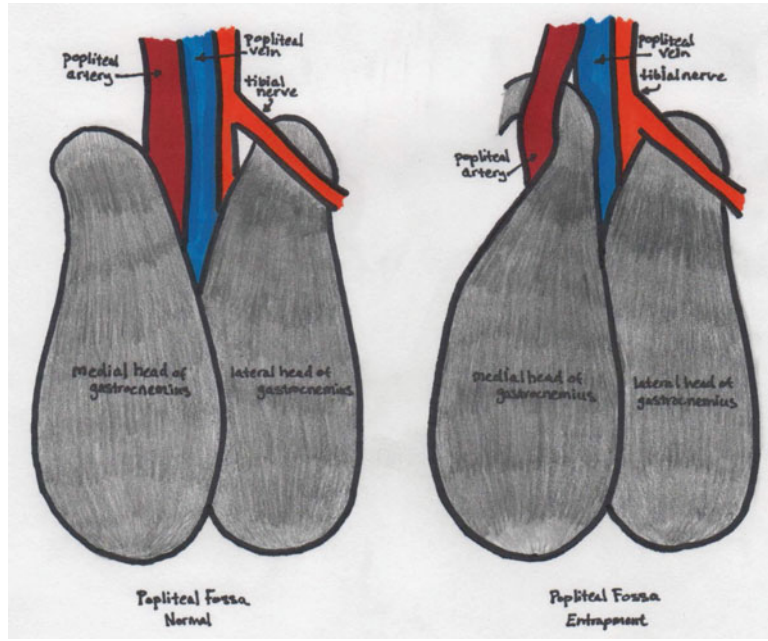

Fig. 27.6 Anatomic popliteal entrapment results from displacement of the popliteal artery by the medial head of the gastrocnemius muscle resulting in popliteal arterial compression. *Left panel*, normal popliteal artery; *right panel*, abnormally displaced popliteal artery

inferior vena cava is formed by the supracardinal veins during which the left supracardinal vein regresses leaving a right sided infrarenal vena cava. In the opposite case, the right supracardinal vein regresses leaving an anomalous left sided infrarenal vena cava. If both supracardinal veins persist, a double inferior vena cava is created [10].

Renal Vein Anomalies

Renal veins develop from the subcardinal veins. The right subcardinal vein gives rise to the right renal vein. The left subcardinal vein forms two branches to the left kidney. One branch runs anterior to the aorta and the other posterior to the aorta. Normally, the posterior vein regresses leaving only the anterior branch to form the left renal vein. If the opposite occurs, with regression of the anterior component, the left renal vein will run posterior to the aorta. If neither component regresses, a left circum-aortic renal vein occurs. In this case, the aorta is surrounded by an anterior and posterior renal vein which may have one or more connections between them. The presence of a circum-aortic renal vein is important to consider when dissecting around the aorta to avoid inadvertent renal vein injury during surgery [11].

Duplicated Femoral Vein

Variations in venous patterns of the extremities are common, the majority of which are without clinical significance. However, variations in femoral vein anatomy can have clinical consequence due to increased risk of deep vein thrombosis (DVT). There are three venous outflow tracts in the mature lower extremity venous network including the femoral vein, the deep femoral vein, and the venous arcades of the sciatic nerve (developed from the primitive axillary vein). Of these three vessels, the femoral vein is normally the dominant outflow tract. In approximately 12 % of people, there is duplication of the femoral vein outflow through either a bifid femoral vein, or through a hypoplastic femoral vein with collateral outflow through an enlarged deep femoral vein or axillary vein. Bilateral cases of duplicated femoral veins are also reported. Duplicated femoral veins have an increased risk of DVT and its associated complications. Detection of DVT in the setting of variable femoral vein anatomy can pose a diagnostic challenge in ultrasound evaluation [12].

Arteriovenous Malformations

Abnormal development of arteries, veins, capillaries or a combination of each results in vascular malformations. Port wine stains are capillary malformations of the dermis causing red or pink lesions to appear on the skin. The lesions occur in 0.1–2 % of newborns. The lesions grow as the child ages. Proposed mechanisms causing capillary malformations include vascular ectasia, lack of neuronal control of blood flow, or over expression of VEGF or VEGF receptors [13]. Genomic studies have identified an association with an activating mutation in the gene encoding guanine nucleotide binding protein G-alpha-q (GNAQ). It is hypothesized this mutation alters cell proliferation through the extracellular signal-regulated kinase (ERK) pathway. These same mutations are also implicated in Sturge-Weber syndrome that includes vascular malformation as a component of a larger syndrome. Sturge-Weber syndrome is a sporadic congenital neurocutaneous disorder that includes a port-wine stain affecting skin in the distribution of the ophthalmic branch of the trigeminal nerve, abnormal capillary venous vessels in the leptomeninges of the brain and choroid, along with glaucoma, seizures, stroke, and intellectual disability. It is thought that the gene mutations causing port wine stains occur 15 later in embryonic development while earlier mutations result in a wider variety of cell types affected causing the syndromic presentation of Sturge-Weber [14].

Parkes-Weber syndrome (PWS) is a disorder that includes a large capillary malformation on an extremity, in addition to soft tissue and bone hypertrophy of the affected limb. The vascular malformations create multiple fast-flow

arteriovenous shunts. Patients with PWS have overgrowth of the affected limb and their clinical course is complicated by the effects of the arteriovenous shunting which cause ulcerations and high output cardiac failure. Similar to PWS, Klippel-Trenaunay syndrome (KTS) is a rare disorder that also includes a vascular malformation. In KTS, patients exhibit a combination of capillary malformations, bone or soft tissue hypertrophy, and lateralizing atypical varicosity. In contrast to PWS, KTS does not have significant arteriovenous shunting. The vascular abnormalities found in KTS generally involve both capillary and venous malformations. There is often a persistence of embryonic veins, most commonly the lateral marginal vein running superficially up the lateral border of the leg. When present, this vein is thickened and incompetent due to the absence of valves. Abnormal veins can also be found involving abdominal or pelvic organs. These malformations are at risk of causing bleeding and thromboembolic events. KTS is important for the clinician, as these patients occasionally seek treatment for varicose veins; due to the underlying hypoplasia of the deep leg veins, treatment of their dilated superficial leg veins is generally contraindicated. The exact developmental cause of KTS is unknown but it is thought to result from abnormal embryonic vascular remodeling, possibly through alterations in VEGF mediated signaling pathways. While KTS is primarily sporadic, familial cases have occurred and genetic studies have also identified an association with mutated angiogenic factor VG5Q [15, 16].

Lymphatic System

The lymphatic system develops from buds off the venous system. Researchers have shown regions of endothelial cells located along the walls of the anterior cardinal veins to express the PROX1 gene. It is from these regions that the lymphatic network arises. Animal models lacking the PROX1 gene fail to develop a lymphatic system. The first step in lymphatic development is the creation of six lymph sacs. These sacs bud off from the cardinal and mesonephric venous systems. These lymph sacs generate additional lymph sacs that spread along the course of developing veins. On both the left and right side, thoracic lymph vessels form from jugular sacs that empty into the venous system at the internal jugular and subclavian junction. In normal development, the left and right side fuse at the thoracic level with regression of the right thoracic vessel to form the left sided thoracic duct draining into the left internal jugular vein. The right sided jugular sac will give rise to the right lymphatic duct. Additional lymphatics develop from the original lymph vessels to form a network of lymphatic channels that all drain into the venous system via either the right lymphatic duct or the thoracic duct. Maturation of the lymphatic vessels is orchestrated through additional cell signaling pathways. The forkhead box protein C2 (FOXC2) plays a critical role in normal lymphatic differentiation into a mature collecting vessel. Ephrin interactions are also important in lymphatic development. Eph-B4 is found on both initial and collecting lymphatics while Eph-B2 is found only on the mature collecting lymphatic vessels [17].

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28 Cardio Vascular Developmental Abnormality of the Human Fetus Appearing Within Second Trimester: Detection and Treatment

Bhabotosh Biswas

Introduction

Fetal Cardio Vascular defects (CVD) have been studied extensively over last two decades. Advancement in imaging technology has helped substantially the Paediatric Cardiologists to gather detailed information of the fetal heart and enabled them to counsel families on "What to expect after delivery".

Earlier the responsibility of treating the babies born with major Cardio Vascular defects were with Obstetrician, Neonatologists and Paediatric Cardiologists. Babies with major CVD used to reach tertiary cardiac care centres in critical condition. As a result mortality used to be unacceptably high.

With the introduction of advanced imaging modalities, the fetal medicine became multi disciplinary. Once the structural cardiac defects are identified by obstetric sonologist, the precise details of CV defects are studied by fetal echocardiographer. In selected subsets of fetuses further details may be gathered with other imaging modalities. Finally a detailed treatment policy can be formulated well before the baby is born. Thus fetal medicine has emerged as a high end speciality for collaborative multi disciplinary care for the fetuses with complex structural CV defects and arrhythmias.

Major congenital heart disease (CHD) is not uncommon with an incidence of 6–12 per 1000 live births [1–4]. Unfortunately sufficient information about major CHD in fetuses is not available in current literature. A study from Belgium [5] reported an incidence of 8.3 % in live and stillborn infants of ≥26 weeks of gestation without chromosome abnormalities. There is likely an even higher incidence in early gestation given spontaneous and elective pregnancy termination [5].

Major CHD is responsible for 40 % of perinatal deaths [3, 4] and more than 20 % of deaths within the first month of life [6]. Unfortunately detection rate of CHD still remains slow. Prenatal detection rate of CHD varies widely ranging from 16 % to 65 % [7, 8]. CHD is wrongly diagnosed even in tertiary care centres by expert teams consisting of Cardiologists and Fetal Sonologists. Poor detection of CHD may be attributed to several factors including gestational age, imaging technique and experience of the Sonologists in addition to maternal and amniotic fluid factors.

Most of the CHDs develop during the first 8 weeks of pregnancy. Many of them continue to develop with advancing gestation [9–12]. Arch obstruction and obstructive lesions of Cardiac chambers may progress from mild to a critical state with advancing gestation. Though Cardiac

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dysrhythmias develop and progress through gestation, Cardiac neoplasm develops during later half of pregnancy only.

Fetal echocardiography is the most valuable tool for detecting major congenital Cardiac defects and has proved itself invaluable for early detection of such lesions. Though majority of the defects can be diagnosed in early pregnancy, few of them are detected only during the later part of gestation.

Fetal echocardiography is usually performed from 18 weeks onwards. Presently first trimester echocardiography is increasingly used for earlier detection of major congenital Cardiac defects. Several studies on first trimester echocardiography detected major CHD at 11–14 weeks in high risk fetuses [13, 14].

A Scientific Statement from the American Heart Association on Diagnosis and Treatment of Fetal Cardiac Disease was published by in Circulation, 2014 [5]. The statement reviewed available literature and commented on the current practice of fetal Cardiac medicine including diagnosis and management [5]. The authors concluded that fetal Cardiac medicine has progressed considerably over the past two decades. Advances in imaging technology and innovations in therapies have been remarkable. Fetal echocardiography has proved to be invaluable in the diagnosis of major congenital Cardiac defects. New technologies including 3D and 4D echo, MRI, fetal Electrocardiography as well as Magnetocardiography are now available. Medical therapy as well as interventions for complex disorders have made substantial changes in outcome for high risk fetuses.

Extensive studies have identified the causes of referral for fetal Cardiac evaluation. Suspicion of a structural abnormality of heart on Obstetric Ultrasonography was the leading cause of referral in 40–50 % of fetuses. A family history of CHD as well as maternal metabolic diseases were other important referring factors.

Fetal echocardiography may be considered when risk is estimated at 1–2 %, although the relative benefit of this additional testing in this population is less clear. When risk approaches that of the general population (≤1 %), fetal echocardiography is not indicated. It should be noted, however, that all fetuses with an abnormal screening ultrasound of the heart should have a detailed fetal echocardiogram by a trained examiner [5].

Common Indications for Referral for Fetal Echocardiography

Several factors, both maternal as well as fetal may be responsible for complicating pregnancies and increasing the risk for major fetal CHD. Following few factors may be mentioned in this context.

- Maternal factors
  - Diabetes Mellitus (DM) affects 3–10 % of pregnancies and causes fivefold increase in CHD compared to the general population of women with DM
  - Medicines taken during pregnancy may be responsible for several congenital defects including Cardiac deformities. A number of teratogens are in clinical use during child bearing age. Use of these medicines during early pregnancy (Cardiogenesis period) increase the risk of CHD. Most important medicines known to cause Cardiac defects are anticonvulsants, vitamin K antagonist, NSAID, Lithium, ACE inhibitors etc.
  - Maternal infections are also responsible for teratogenesis including defective formation of heart.
  - Infertility treatment adopting assisted reproductive technology have also been held responsible for increase in fetal CHD.
  - Family history of CHD has also been evidenced to increase fetal CHD.
- Fetal factors
  - Suspected anatomical defect of heart in obstetric ultrasound warrants further evaluations by fetal echocardiography for early detection and planning of therapy for fetal CHD.
  - Fetal arrhythmias may be associated with CHD. Fetal bradycardia resulting from abnormal AV conduction is more likely to be associated with CHD compared to fetal tachycardia.

- CHD may be associated with non Cardiac malformations as well.
- Increased nuchal translucency on first trimester screening is an important indicator to suspect fetal CHD.
- Abnormalities of umbilical cord and venous system is associated with higher incidence of CHD.

Fetal Echocardiography

A paragraph regarding Fetal echocardiography at this juncture may help further understanding of fetal CHD and enable the physician as well as family to take a right decision in time

Fetal echocardiography remains the primary tool for the evaluation of major CHD, though its scope may be controversial in some cases. Current fetal echocardiography has included a wide variety of modalities which enable to study the fetal Cardiac structure and functions more precisely and elaborately. Various components of fetal echocardiography have been recommended by different authors.

Fetal echocardiography for screening of CHD of high risk pregnancies are usually performed between 18th and 22nd weeks of gestation. However this timing may fail to identify Cardiac disorders that progress from subtle pathology in midterm to major CHD in full term. Detection of abnormal finding on routine Obstetric Ultrasound usually indicates fetal echocardiograph for detailed Cardio-Vascular evaluation. After detailed initial evaluation, follow up fetal echocardiogram has a specific role for detecting progressive fetal CHD. Addition of color Doppler imaging provide invaluable information regarding valve function and vessel patency.

Prenatal Counseling has a specific role in the work up of fetal CHD.

Once an accurate diagnosis of fetal CHD is made after proper evaluation, it must be communicated to the family members providing proper management plan and a truthful picture of prognosis. This helps the parents to take appropriate decision about termination of pregnancy or about palliative care to be given to the baby after birth.

Therapeutic Options for Fetal Cardio: Vascular Defects

Institution of appropriate therapy for congenital heart defects before birth is now a reality.

Improved accuracy in diagnosis of Cardiac defects in utero helps to make therapeutic plan well in time. Varieties of therapeutic options are now available for treating various fetal Cardiac defects.

Followingtherapeuticoptionsarecurrentlyavailable-

(a) Maternal administration of medication with transplacental transfer to the fetus
(b) Minimally invasive fetoscopic guided techniques/
(c) Open uterine fetal surgery

However fetal therapy is still in infancy. Risk to the mother as well as fetus are real concerns for current day fetal therapy.

Certain Cardiac lesions are now considered suitable for fetal surgery. Aortic Stenosis (AS) with evolving hypoplastic left heart syndrome (HLHS), HLHS with restrictive or intact atrial septum, pulmonary atresia with intact ventricular septum, mitral valve dysplasia with MR and AS etc. are few conditions in which fetal Cardiac surgery has proved useful.

Therapy for Fetal Cardiovascular Defects before Birth

(a) Maternal administration of medication with transplacental transfer to the fetus –

This modality has been established as a therapeutic option specially for the following clinical conditions—

Fetal Arrhythmias

Fetal Bradycardia

The cause and mechanism of fetal bradycardia dictates treatment strategy. It is usually close observation for signs of fetal distress. Decisions on early delivery and resultant prematurity must be weighed against therapies available. Risk of

both mother and fetus needs consideration. If bradycardia persists, it should be evaluated thoroughly after delivery.

AV Block

Treatment of CHB depends on several factors including the origin, the ventricular rate, and the degree of heart failure. Regardless of the origin, the use of β-sympathomimetics (terbutaline, salbutamol, isoprenaline) to augment fetal ventricular rates when <55 bpm has been reported. Terbutaline is well tolerated, although maternal resting heart rates of 100–120 bpm and benign ectopy may be encountered [15].

Fetal pacing does not improve survival [16] and still experimental and therefor not recommended.

Fetal Tachycardia

Fetal tachycardia is a rare but an important cause of intrauterine fetal nonimmune hydrops, premature delivery, and perinatal morbidity and mortality. Treatment of fetal tachycardia depends on its cause. Conversion to 100 % sinus rhythm is not the target but to achieve sufficient sinus rhythm to allow reasonable ventricular function.

Irregular Rhythm

Is found in 1–3 % of all pregnancies and is usually harmless of pregnancy.

Anti Arrhythmic Drugs

Relatively high doses are required during the second and third trimesters of pregnancy due to increased maternal circulating blood volume and renal clearance. Maternal transplacental treatment is initiated in the hospital and in most cases, oral antiarrhythmic agents are continued.

Congestive Heart Failure of Fetus

Transplacental digoxin may be considered, although its usefulness is doubtable

(b) Minimally invasive fetoscopic guided techniques/interventions

Cardiac Catheter Intervention in Fetus

Cardiac lesions that are amenable to fetal intervention are distinctive in that they can lesion progressing rapidly to severely during gestation myocardial damage need fetal catheterisation.

Fetal cardiac intervention usually leading to alter the natural history of the disease and results in an improved state at birth reducing short- or long-term morbidity or mortality.

Following Cardiac Lesions May Be Amenable to Fetal Intervention

AS with Evolving HLHS

Severe AS in early and mid gestation evolving into HLHS at birth, are suitable candidates for such therapy [17–26].

HLHS with Intact Atrial Septum

HLHS with intact atrial septum have very high mortality and morbidity even after neonatal survival [27]. The fetus with this condition is stable in utero, [27, 28] the newborn becomes critically ill immediately after birth and succumbs to hypoxia, acidosis, and pulmonary oedema. If diagnosed prenatally, a planned delivery with urgent catheterization and balloon dilation or stent dilation of the atrial septum may be done. However favourable, outcome is not expected [29, 30].

Mitral Valve Dysplasia with Mitral Regurgitation and Aortic Stenosis

Fetal cardiac intervention to open the aortic valve may be useful but may end in gross aortic regurgitation [31] Opening of the atrial septum to decompress the left atrium may also help [32].

Pulmonary Atresia with Intact Ventricular Septum

Fetuses with PA and IVS may be candidates for fetal cardiac intervention. It may prevent the need for single-ventricle palliation after birth [33–35].

(c) Open uterine fetal surgery

Surgical Techniques

Fetal surgery may be performed with hysterotomy and exposure of the fetus or through laparoscopy.

Fetal surgery may be suitable in the following conditions ---

Giant sacrococcygeal teratomas large congenital cystic adenomatoid malformations with signs of hydrops, severe congenital diaphragmatic hernia, and meningomyeloceles.

Fetal echocardiography may be useful for assessment of cardiac function and fetal circulation before, during, and after surgery.

Left ventricular hypoplasia may be associated with congenital diaphragmatic hernias resulting from ventricular compression or diminished filling secondary to pulmonary hypoplasia and decreased pulmonary venous return [36].

Open Fetal Surgery

Surgical repair of CHD in a fetus may offer greater benefit than over postnatal repair in selected patients.

However optimal techniques and method of selection of proper candidates, are yet to be established.

In animal models, Cardio Pulmonary Bypass in the fetus results in significant placental dysfunction [37].

Impacts of Fetal Surgery

Need for ventricular shunt procedures was found to be reduced after Open fetal surgery for meningomyelocele repair done before 26 weeks of gestation. Improvement in motor outcome at 30 months of age were also comparable in this subset of cases.

Resection and acute relief of cardiac tamponade in congenital cystic adenomatoid malformation was found to result in acute mismatch in volume with filling impairment along with ventricular dysfunction.

Potential Benefits of Perinatal Management of CHD in Fetuses

Provides sufficient time for extensive prenatal counselling and allows better prediction of the clinical course in utero as well as during the circulatory transition occurring with delivery.

Benefits of Prenatal Diagnosis and Perinatal Management

Critical neonatal CHD diagnosed prenatally, affects neonatal morbidity and mortality. Fetuses with ductal-dependent pulmonary or systemic blood flow require institution of prostaglandin infusion soon after birth to prevent ductal closure. Because the ductus arteriosus does not close at delivery, these newborns are not expected to be compromised in the delivery room [38–40] and can be stabilized by collaborative team efforts of neonatologists and pediatric cardiologist before transfer for surgical intervention.

Ductus dependent CHD diagnosed prenatally, have been shown to be less compromised preoperatively than infants diagnosed after birth. Improved arterial pH, improved oxygenation, less myocardial dysfunction, and less end-organ disease such as necrotizing enterocolitis and renal injury have been observed in cases diagnosed prenatally [41, 42].

Prenatal diagnosis of CHD may improve fetal and perinatal outcome by reducing intrauterine heart failure, as it helps to initiate intrauterine medical therapy and optimization of perinatal management strategies, including early delivery, if necessary.

Delivery Planning

For assuring optimal benefit for fetal CHD diagnosed prenatally, perfect coordination of intrapartum care is essential between Obstetric, Neonatal, Cardiology including Interventional and Electrophysiology, Intensive care, as well as Cardiac Surgery. Overall neonatal condition and surgical outcomes are improved by delivery in close proximity to a Cardiac center with the resources needed to provide medical and surgical interventions for infants with specific major cardiac defects [42–46].

Delay of elective delivery until 39 completed weeks of gestation has been shown to improve neonatal outcomes [47]; however, waiting beyond 42 weeks has been shown to be detrimental [48].

Recommendations for Transitional Care

Fetal diagnosis of high risk complex CHD prevents the postnatal hemodynamic instability during transition at delivery [42, 44, 49–53]. Usually two major systems-Cardio Vascular and Respiratory, play a significant role in successful fetal-neonatal transition. In case it is apprehended that one or both of these systems cannot transi-

tion normally, a specialized plan of care needs to be organised for a favourable outcome.

Left-to-right shunt lesions (Ventricular septal defects and Atrial Septal Defects) without severe Pulmonary Arterial Hypertension (PAH) are generally stable. Mild valve disease with normal cardiac function are usually asymptomatic in the neonatal period, although progression of valve dysfunction may occur rapidly in some cases [40, 54–57]. For minimal-risk neonates, specialized care is not recommended in the delivery room.

Fetal Arrhythmias

Fetal arrhythmias may require intervention- electric conversion to sinus rhythm or administration of antiarrhythmic drugs, in the delivery room itself, specially if the delivery is due to impending heart failure, hydrops, or fetal distress [58–60].

Fetal medicine has already emerged as a high end speciality. Role of fetal medicine in the field of major Cardio Vascular disorder occurring in first and second trimester of gestation has been well established. Advanced imaging modalities specially fetal echocardiography has a major role for in-utero diagnosis and planning multispecialty well co-ordinated collaborative therapy for fetal complex cardio vascular disorders. Though extensive studies during last two decades have contributed tremendously to enrich fetal medicine, the super speciality has to go a long way to offer safe remedies for most of the complex cardio vascular disorders diagnosed during first and second trimester of gestation.

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Part IX

Fetal Neurological Development: Up to Second Trimester and Its Implication in Adult Neurodegenerative Diseases

29 Implications of Foetal Development and Neurodegeneration in Adult Life

Biman Kanti Ray

Introduction

The role of early life in health and development of an individual is very important. Developmental period is not controlled by a strict, hard-wired genetic programme. Now this period is considered as a plastic phase which allows the organism to adopt to changes in the environment, specially during early development when cells are differentiating and tissues are developing.

Environmental Factors in Neurodegeneration

Interactions between genes and environmental factors during developmental period play an important role not only in human behavior but also in susceptibility to disease [1]. Environmental factors such as foods, metals, pollutants, microorganisms in early life interact with the specific loci thereby modifying their expression and play a direct or indirect role in brain health [2–8]. Epidemiological and animal based studies have suggested that adverse prenatal and postnatal environmental conditions increase the risk of neurodegenerative diseases [9–13].

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Human Studies

Studies have revealed that chronic placental insufficiency may result in foetal hypoxemia which leads to synaptic dysfunction that triggers neurodegeneration in neonates [14]. Maternal hormonal disturbances also affects foetus adversely. Well-designed human studies showing the effect of in-utero exposure on neurodegeneration are limited. Most studies are either retrospective or short duration prospective.

Animal Studies

Animals provide an excellent model for prospective study of early life exposure due to shorter life span and better follow-up. Perinatal hypoxia results in retinal ganglion cell degeneration in rat models and brain damage in the rabbits and the developing brain is more vulnerable than the adult brain to the same insult [15–17]. In an interesting study, pups of female exposed to bacterial endotoxin, Lipopolysaccharide (LPS) during pregnancy showed loss of dopaminergic neurons enhancing susceptibility to Parkinsons disease [27].

The effect of following environmental factors during in-utero and developmental stages of life have been studied and based on these studies, various models have been proposed.

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Dietary Exposure

Foetal development is dependent on nourishment provided by the maternal system and thus deficiency or excess of any nutritive supplement to the mother adversely affects the foetus [18].

Human Studies

Vitamin B12 deficient diet to the mother during pregnancy adversely affects the myenilation in nervous system of offspring [19]. Inappropriate micronutrient supplementation in mother affect the level of anti-oxidant enzymes in the offspring, thus increasing the risk of neurodegenerative diseases [31].

Animal Studies

Correlation between maternal diet and foetal neurodegeneration has been reported in several animal studies. Maternal folate depletion results in epigenetic changes in the off-springs leading to neurodegeneration [20]. Elevated Homocysteine level in mother was shown to increase oxidative stress in pups brain leading to apoptosis [21]. High dose of Iron in neonatal stage has been shown to result in neurodegeneration of midbrain at later age [32].

Metal Exposure

Blood-Brain-Barrier and formation of metal-protein complex protects the mature brain tissue from foetal toxicity. In foetal brain this sequestering mechanism is absent.

Human Studies

Metals such as aluminium, zinc, iron, copper and mercury have been shown to be associated with neurodegenerative diseases [33]. Metals like zinc and copper are believed to result in A-beta aggregation [22]. Lead exposure in first trimester of pregnancy results in increased amplitude of a and b waves in 7–10 year old children. High level of Mercury and Lead in umbilical cord blood due to prenatal exposure impaired the visual processing in children after 11 years [23].

Animal Studies

Long – term potentiation (LTP) is considered the key mechanism underlying information storage and memory formation. LTP is dependent on presynaptic increase of neurotransmitter release and enhanced function of glutamate receptor at the post synaptic end. NMDA receptor function has been found crucial for the LTP induction in hippocampus. Exposure to metals such as zinc, copper, aluminium and lead is associated with a negative effect on LTP during developmental stage. Various studies show that the lead exposure in developmental stages results in the increased level of beta amyloid in brain causing Alzheimer in later age [24, 25].

Pesticides

Pesticides including insecticides (organophosphates, organochlorines, carbamates), herbicides and fungicides enter the body through respiratory tract, gastrointestinal tract, dermal contact and rarely via ocular exposure.

Human Studies

Pesticides get accumulated in the body and change the gene expression profile in exposed tissues through epigenetic changes. Pesticide exposure has been shown to result in neuronal loss, cognitive impairment and motor dysfunction [26].

Animal Studies

Exposure to dieldrin and paraquet during gestation and lactation has been reported to affect the dopaminergic responses in off-springs and leads to Parkinsons disease in adult life [28].

Lifestyle, Smoking and Drug Abuse

Healthy lifestyle prevents disease whereas bad habits increase susceptibility to disease. Exercise particularly aerobic exercise has a positive impact on brain functioning.

Human Studies

Childhood aerobics increases the resilience of the brain in later life. Similarly, the association of caffeine, smoking and alcohol consumption has been well documented in neurodegenerative diseases [29, 30].

Animal Studies

One study showed that pups born to mothers undergoing low intensity treadmill exercise during pregnancy have more hippocampal cell survival [33]. Similarly, pups performing treadmill exercises at postnatal day 21–60 showed enhanced spatial memory as compared to controls. Female rats exposed to methamphetamine at gestational stage have shown smaller optic nerve diameter than controls [33].

Mechanism, Hypothesis and Models

Epigenetics

Epigenetics is an emerging field that focuses on the mechanisms that alter the function of genes independent of the primary DNA sequence [33, 34]. Thus, independently of the genotype, different epigenetic profiles may result in different phenotypes. Thus, each individual has one genome, but multiple epigenomes. Mechanisms such as DNA methylation, histone tail modification and regulation by non-coding RNAs are responsible for these alterations. These epigenetic marks, particularly if they are near the promoter region of functional genes, can influence transcription by altering access of transcriptional machinery to the DNA. DNA Methylation may occur through recruitment of Methyl-CpG binding domain proteins while histone modifications can change chromatin conformation between open and closed states, resulting in altered availability of DNA for transcription. The non-coding RNA referred to as microRNA is believed to act at post transcriptional stage thereby exerting epigenetic regulation of such changes. Recent evidence suggests micro RNAs play role in DNA Methylation. Alterations in gene expression patterns induced by these mechanisms may be more frequent than genetic mutations. It generally takes into account the gene and environment interaction such that these changes are inherited. Such epigenetic modifications can be passed from one cell generation to next and in some case, can be transgenerationally transmitted. Recent evidence supports that adult neurogenesis is under intensive regulation by epigenetic mechanisms [34]. These changes can be cell, tissue and sex specific and time dependent. The epigenome is altered by environmental signals, not only during the period of exposure but even later in life. The most sensitive window for epigenetic effects varies among different tissues and may extend into early childhood or beyond for some tissues such as brain.

Baker Hypothesis or Foetal Basis of Adult Disease (FeBAD)

According to this hypothesis, adult diseases are consequences of foetal adverse conditions [18]. Although Baker's work was mainly confined to cardiovascular diseases, this hypothesis fits well to other diseases too. Early life origins of later life disease is often referred to as programming [35]. Hales and Baker defined programming as permanent or long term change in the structure or function of an organism resulting from a stimulus or insult acting at a critical period of early life. The adult outcome of early life programming are dependent on the nature of insult, the timing in which insult occurs and the duration of insult. The insult adversely affects the organs that undergo rapid growth in the offspring at the time of the insult occurring.

Developmental Origin of Health and Disease (DOHaD)

This model is a modified version of FeBAD which postulated that postnatal period of development also plays an equal role as foetal life in health and changing environmental factors affect the patterns of diseases.

Predictive Adaptive Response (PAR)

Gluckman and Hanson have suggested that foetus undergoes adaptations during gestation or early postnatal period based on the predicted postnatal environment [35]. The foetus predicts the extra-uterine environment from intra-uterine conditions and makes changes for its better survival. However if the predictive environment does not match the actual environment, then the programmed adaptations will become detrimental.

LEARn Model (Latent Early Life Associated Regulation)

Lahiri et al. suggested the role of early environmental factors in disease etology especially with respect to Alzheimer's disease [36]. LEARn model describes the environmental exposures such as nutrition, head trauma, metal exposure and life style as hits. They postulated that all neurodegenerative disorders come under the category of n hit latent model and early life environmental factors lead to epigenetic perturbations in the genes but do not result in any disease symptom. When there is a second trigger, the disease develops and the time interval between first hit and disease onset is called latency period. Genes are divided in two groups (1) which respond late in relation to early life responses (LEARned) (2) Which do not respond (unLEARned).

Epigenetic Regulation of Adult Neural Stem Cells

Since the early 1900s, regenerative mechanisms in adult CNS have been thought to be very limited. The predominant repair mechanisms considered in the CNS were post-mitotic such as sprouting of axon terminals, changes in receptor expression and synaptic reorganization. However, recent findings have clearly demonstrated that new neurons are indeed born from neural stem cells in restricted regions of adult mammalian CNS throughout life [34].

What Is Neural Stem Cells (NSC)?

NSC are defined as cells present in some areas of adult brain that have the capacity to produce new, functional neurons that are added to existing brain circuits.

During development of the mammalian CNS, neurons and glia arise from multipotent neural stem cells in which neurons are generated first, primarily during the embryonic period followed by glia, the majority of which differentiate after most neurons are born.

Although NSC may exist along the entire adult neuroaxis, adult neurogenesis has consistently been found only in subventricular zone (SVZ) of the lateral ventricle and in subgranular zone (SGZ) of the hippocampal dentate gyrus. Some authors have suggested that neurogenesis occurs also in other regions of CNS such as the cortex and substantia nigra. Neurogenic niches are unique tissue microenvironments that are permissive to the presence of NSC in the adult brain [34]. Steps involved in neurogenesis are: proliferation of neural stem cells, generation of a rapidly amplifying progenitor cell, differentiation in immature neuron, migration to the final location, growth of axon and dendrites, formation of synapses with other neurons in the circuit and ultimately maturation into a fully functional neuron. The fundamental difference between developmental and adult neurogenesis is that new adult neurons undergo these processes in an already mature environment and therefore have to integrate into preexisting circuits. Multiple intrinsic and extrinsic factors have been identified e.g. growth factors, morphogens, transcription factors and cell cycle regulators which control neurogenesis. A combination of niche signals and cell intrinsic programmes control the transition from an undifferentiated NSC state to a progenitor cell committed to the neuronal fate. Epigenitic

mechanisms are likely key players within these signaling networks, as DNA methylation, chromatin remodeling and small non-coding RNAs from the microRNAs superfamily are required for fine-tuning and coordination of gene expression during adult neurogenesis.

Is Neural Stem Cells in the Adult CNS an Endogenous Source to Repair?

During the past decade, the progress in the field of stem cells has fuelled our hopes that endogenous stem cells can be mobilized to replace dying neurons in neurodegenerative diseases and cure the currently incurable diseases by cell replacement [37]. The use of endogenous sources for cell replacement offers several advantages. Many ethical concerns and political restrictions regarding the use of foetal tissue and embryonic stem cells do not apply for endogenous sources and immunological reactions are avoided. However, for successful use of endogenous neural stem cells for cell replacement, there are multiple specific challenges and problems to be overcome.

Repair from Adult Stem Cells from Neurogenic CNS Regions

Stem cells in neurogenic areas respond to injuries. There is proliferation of precursor cells in SVZ and SGZ shortly after seizure and ischaemia. But it is premature to conclude that increased neurogenesis equals the regeneration of the compromised circuit. First, there is no direct evidence that the new neurons are replacing neurons that degenerate owing to injury. Second, the generation of new neurons in neurogenic areas has not been proven to be causally linked to functional recovery. Third, lesion induced neurogenesis might also contribute to pathological alteration of function due to aberrant migration, network reorganization and altered physiological properties of the newly generated ectopic neurons. For injury induced neurogenesis to be advantageous, the new neurons have to integrate appropriately to the injured neuronal circuit, display functional properties similar to the lost neurons and be generated in number that are comparable to the number of lost neurons.

Repair from Adult Stem Cells in Non-neurogenic Regions

Several studies reported increased cell proliferation and generation of immature neurons in normally non-neurogenic region of CNS following focal stroke by occlusion of middle cerebral artery.

Implications for Treatment of Human Diseases

There are many diseases with different pathophysiologies affecting diverse regions of human CNS. We are far from understanding the processes regulating physiological neurogenesis and lesion-induced neurogenesis and it is too early to comment on the suitability of cell replacement from endogenous stem cells in the context of specific diseases. Some specific aspects of CNS diseases that may have a major impact on the potential and strategy for cell replacement therapy include:

Injury/Disease timing—Whether the CNS disease is acute or death of neuron occurred over extended time-period e.g. Neurodegenerative diseases.

Disease location—Neuronal cell replacement has been primarily observed in regions close to Lateral Ventricle and has been ascribed to neurons that have origin from SVZ.

Extent of cell loss—Greatly influence whether functional regeneration from endogenous stem cells can be achieved.

Genetic basis of disease—Genetic disease will also be present in the new neurons derived from endogenous stem cell population. It is not known whether this will lead to degeneration of the newly generated neuronal population.

Effect of the disease on the environment—Injury or disease may create a milieu that is hostile to the generation of new neurons e.g. acute stroke increase the presence of scar inducing signals that have an inhibitory effect on endogenous stem cells. Disease like stroke affect not only neurons but also glia. Glial cells create the necessary milieu for neuronal

function. It is therefore very important to focus not only on the generation of new neurons but also on the cellular environment that supports the maturation, survival and function of new neurons.

Therapeutic recruitment of endogenous Neural Stem cells: some unanswered questions— Are neural stem cells truly present throughout the neuroaxis? What degree of plasticity do the adult stem cells have and do the adult neural stem cells in different regions display the same degree of plasticity?

Till now, it has been demonstrated that adult neural stem cells can give rise to the functional neuronal phenotypes of the olfactory bulb and the hippocampal dentate gyrus. But it remains unanswered whether endogenous neural stem cells can also generate other neuronal phenotypes such as functional dopaminergic neurons of the substantia nigra pars compacta or motor neurons of ventral horn. Finally, we cannot assume that our findings in animal models are readily translatable into the human system. We still have to go a long way before we can realistically propose cell replacement using endogenous stem cells as a potential source.

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Part X

Fetal Neuropsychiatric Development: Up to Second Trimester

Very Low Birth Weight Babies and Their Mental Health Outcome

30

Pradeep K. Saha

Introduction

Preterm birth and low birth weight babies are an emerging public health concern in India due to several factors as improvement of early neonatal services and booking of pregnancies in India, where the survival rates of the babies of these categories are increasing. Improvements in antenatal care and advances in neonatal medicine have resulted in increased survival of infants, in particular those of very low birth weight (VLBW) (<1,500 g) and extremely low birth weight (ELBW) (<1,000 g). If simple gain in life years is taken as a standard, then neonatal care is the most successful discipline in medicine today [1]. The psychological development and quality of life of VLBW and ELBW children has become an increasing focus of recent research [2]. The focus of this chapter will be the outcomes of the survivor babies of the birth-weight range of 1,000–1,500 g.

The Institute of Medicine Committee on Understanding Premature Birth and Assuring Healthy Outcomes Board on Health Sciences Outcomes, Washington, DC, USA (IOM) report describes prematurity not as a single disease, but a common complex condition that results from multiple gene–environmental interactions that lead to several final pathophysiological pathways to birth before 37 weeks gestation [3]. The many biological and environmental risk factors for preterm birth, including biological factors, maternal medical conditions, genetics, environmental exposures, assisted reproductive technology, behavioural and psychosocial factors, and neighborhood social characteristics, vary among populations. A prior preterm birth is the strongest risk factor. Pathways to preterm birth include inflammation (resulting from environmental exposures to antigens and infections, as well as foetal and maternal immune responses), uterine distension (e.g. multiple gestations) and deteriorating foetal or maternal health (e.g. preeclampsia). For over 50 years, preterm outcome studies have reported higher rates of cerebral palsy, intellectual disability and sensory impairment in preterm survivors as compared with peers born at term [3, 4]. The focus of more recent studies has shifted to describing school and behaviour problems (e.g. minor neuromotor dysfunction, specific learning disability and language, visual-perceptual and attention deficits), and the contribution of preterm complications and their treatments to neurodevelopmental outcomes.

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An Overview of the Different Outcomes of the 1,000–1,500 g Birth Weight Survivors

Neurodevelopmental Outcomes

Much of the variation in reported rates of neurodevelopmental disabilities may be due to differing methodologies, but the challenge is in discerning how much is due to neonatal intensive care unit (NICU) treatment strategies [3–9]. Commonly used terms that describe study samples (e.g. very low birth weight) have become so confusing that exact descriptions (e.g. infants with birth weight below 1,500 g) will be used here. Variations in length of follow-up, success in tracking subjects, evaluation measures and diagnostic criteria influence disability rates, as demonstrated in a German study that annually evaluated children with birth weight below 1,000 g with neurological examinations and a variety of psychometric tests (based on age) [10]. At 6–10 years, 17 % had major impairment (cerebral palsy, intelligence or developmental quotient below 70, blindness or intractable epilepsy), 42 % had minor impairment (neuromotor abnormalities, intellectual or developmental quotients of 70–84, socio emotional problems, or language, gross motor, fine motor, visual, auditory, or attention deficits), and 41 % were normal. Only 1 of 12 infants with cerebral palsy had not been diagnosed at 18 months, but cognitive impairments were more difficult to identify. The proportion of assessments that concurred with 6–10-year outcomes increased with length of follow-up: only 49 % at term (i.e. 40 weeks postmenstrual age), but 59 %, 68 %, 70 % and 70 % and at 1, 2, 3 and 4 years, respectively.

Motor Outcomes

Although many preterm infants demonstrate neuromotor abnormalities on examination, most do not develop cerebral palsy [3, 7, 10–18]. Rates of cerebral palsy in infants born after 1990 range from 4 % to 12 % for survivors with birth weight below 1,000 g, 6–20 % for infants born before 27 weeks gestation, and 21–23 % for infants born before 25 weeks gestation [3]. In children born 1994–1997 in Northern Ireland, cerebral palsy prevalence was 1.2 per 1,000 live births for birth weight over 2,499 g, 11.3 for birth weight 1,500–2,499 g and 44.5 for birth weight below 1,500 g [16]. Mortality is high in infants with birth weights below 1,000 g, so cerebral palsy developed in 47 per 1,000 live births and 99.5 per 1,000 (9.9 %) survivors. Although they constitute only 1 % of births, 22 % of children with cerebral palsy were born with birth weight below 1,500 g. An increase in cerebral palsy with decreasing birth weight and gestational age category is a consistent finding in preterm outcome studies. This finding is not limited to extremely preterm infants. Marret et al. [14] report an increase in cerebral palsy with each preterm week: 0.7 %, 3.7 %, 4.1 %, 8.7 % and 6.3 % in children with gestational age 34, 33, 32, 31 and 30 weeks, respectively ($P<0.01$). In a Swedish study, children born at 32–36 weeks gestation had a six times higher prevalence of cerebral palsy than children born at term (7 per 1,000 vs. 1.1 per 1,000 live births) [18]. Being born just a few weeks preterm confers enough vulnerability and complications of prematurity to increase both mortality and morbidity rates over those of full-term neonates. A recent report from the American Academy of Paediatrics Committee on Foetus and New-born proposes guidelines for evaluating and managing 'late preterm' infants born at 34–36 6/7 weeks gestation [19]. Many children born preterm demonstrate mild fine or gross motor delay, mild but persistent neuro-motor abnormalities (e.g. asymmetries, tight heel cords), motor planning problems and/or sensorimotor integration problems that lead to functional impairments (e.g. tying shoelaces), academic difficulties (e.g. writing), and social–emotional problems (e.g. poor self-esteem and peer relationships) [3, 7, 17, 20, 21]. In an excellent Australian study of 8–9-year-olds, children (especially males) with birth weight below 1,000 g or gestational age below 28 weeks with developmental coordination disorder (9.5 % vs. 2 % in normal birth weight controls, $P<0.001$) had more problems with cognitive function,

academic achievement, and adaptive behaviour [20]. In a Swiss study of 6-year-olds, children with birth weight below 1,500 g had low scores on multiple motor tasks (especially quality and speed of movements) [21]. Scores correlated with brain injury on neonatal ultrasounds and neurological abnormalities on examination at age 6, but even preterm children with no neurological abnormalities had lower scores than the referent population. A British study of 6-year-olds noted poorer performance on a variety of motor tasks in children with gestational age below 26 weeks compared with full-term controls, as well as a higher prevalence of left-handed preference (28 % vs. 10 %), overflow movements during motor tasks, sensorimotor difficulties and visuospatial problems [17]. These problems adversely influenced the children's classroom performance as rated by their teachers.

Cognition and Academic Achievement

Recent studies have not only confirmed that children born preterm have more cognitive impairments and academic difficulties than full-term controls, but they also suggest that these are more common than motor, visual or hearing impairments [3, 11, 15, 17, 22]. In a study of 2-year-olds, 54 % of children born before 27 weeks gestation had a Griffith Mental Developmental Quotient greater than two standard deviations below the mean; only 40 % had normal cognitive abilities [12]. A study comparing children born with birth weight below 1,500 g during three epochs found no significant changes in cognitive tests from 1982 to 2002, but 20–24 % had Bayley Mental Developmental Index scores below 70 [15]. The French study of children born at 30–34 weeks gestation found cognitive impairment to be the most common disability, and with increasing gestational age, mean cognitive scores and proportion of children with scores above 85 improved (from 94 to 98 and from 65 % to 76 %, respectively) [14]. Difficulties with reading and spelling increased with decreasing gestational age (and birth weights) in a Danish study of 11–13 year olds [22]. They found significant differences between children born at 33–36 weeks (and at 37–38 weeks) compared with children born at 39–40 weeks gestation. In addition to birth weight and gestational age, factors associated with cognitive outcomes include neuroimaging evidence of brain injury, neuromotor abnormalities on exam, male gender and some factors related to severity of neonatal illness or chronic lung disease [3, 12, 14, 22, 23]. Vlastos et al. [24] found that foetuses with absent foetal heart rate reactivity prior to delivery had higher risks of brain injury and low Bayley scores. In a ventilation trial of children born before 28 weeks gestation, factors associated with lower vocabularies at age 2 included severe neurosensory impairment, male gender, length of hospital stay, and weight at 12 months [23]. Sensory impairments Visual and auditory impairments are associated with preterm birth, and the most immature and sick preterm infants have the highest risks [3, 12, 14, 25–27]. In a study of caregiver-reported outcomes of preschool children, 6 % of children born before 28 weeks gestation had moderate to severe visual impairment and 4 % had moderate to severe hearing impairment, compared with 0.5 % for vision and hearing in children born at 28–32 weeks gestation and 0.3 % for each in full-term controls [28]. Severe visual impairment occurs in 1–3 % of children with birth weight below 1,000 g and 2–6 % in children with gestational age below 27 weeks [3, 12, 14]. Although as many as 6 % of children with gestational age below 26–27 weeks have severe to profound hearing impairment, many function well after early placement of cochlear implants [3, 12].

Adult Outcomes

Many young adults with birth weight below 1,000–1,500 g finish high school, find employment, marry and live independently [3, 29]. Nonetheless, some studies have found that more young adults with birth weight below 1,500 g leave school early and fewer go on to college and graduate from 4-year courses. Although more young adults born preterm demonstrate shy and

withdrawn behaviours, they have fewer risk-taking behaviours (e.g. use of alcohol or illegal drugs, delinquent behaviours, teenage pregnancies) than full-term controls. In an impressive Australian 31-year follow-up study of offspring of mothers in an antenatal steroid trial, there were no differences between 126 adults born preterm (mean 34 weeks gestational age) and 66 full-term controls in marital status, educational attainment, socioeconomic status, cognitive functioning, working memory, attention or psychiatric symptoms [30]. They found that preterm birth was associated with fewer symptoms of depression and greater satisfaction with respect to general health, pain, and social functioning. A Canadian study of young adults with birth weight below 1,000 g reports that despite more functional cognitive, mobility, and self-care limitations, they considered themselves to have a similar quality of life as their full-term peers [31].

Depression and Infants Born with Birth Weight 1,000–1,500 g

Over the past two decades a growing body of research has linked low birth weight to a variety of adult health outcomes, first and most compellingly for cardiovascular disease [32–34], but also for hypertension and type II diabetes [35, 36] and less clearly some mental disorders [37, 38]. These findings support what is variously termed the 'Barker hypothesis', 'foetal origins hypothesis' or 'developmental origins of adult health and disease hypothesis' [39]. It describes developmental plasticity in the foetus and young infant as a mechanism for permanently adjusting or 'programming' aspects of its physiology in response to the intrauterine and post-natal environment. This may provide adaptive fitness: exposure to poor nutrition in early life may programme glucose–insulin metabolism to maximise fitness by increased insulin resistance. The survival advantage gained in better handling early malnutrition may come at the cost of increased susceptibility to chronic disease in later life [40, 41]. Population-based cohort studies have presented mixed results and there is currently no consensus whether low birth weight is associated with depression in later life [42–45]. High-risk populations, including those exposed to famine, provide particularly strong evidence of an association. For example, Brown et al. [46] found that men and women exposed to the Dutch Hunger Winter of 1944–1945 during their second or third trimesters were at significantly increased risk of affective disorder [odds ratio (OR) 1.54, 95 % confidence interval (CI) 1.12–2.13] for exposure in the second trimester. However, the interpretation of this finding is complex and difficult to generalise to less extreme conditions. Famine no doubt represents a complex cluster of exposures, which in the perinatal period could include extreme material deprivation, maternal ill health and severe stress. There is a need to consolidate the epidemiological data from population studies under more normal circumstances. In an exhaustive meta-analysis of 18 studies by Wojcik et al. [47], demonstrated a weak association between low birth weight and later depression, including psychological distress, which became non-significant after correction for probable publication bias. The same was true if the analysis was restricted to studies examining depression only. Of the 13 studies that reported null findings in relation to their predetermined definitions of low birth weight and depression outcomes, the majority (n = 11) emphasised 'positive' findings in their abstracts, such as subgroup analyses or secondary exposures. Only two of these null studies did not prioritise other positive findings [45, 48]. This suggests a preference for authors and journals to report positive findings while null findings are often downplayed, a phenomenon widely recognised [49]. They concluded that there was evidence of reporting and publication bias and that their meta-analysis should be interpreted as indicating no compelling association between low birth weight and adult depression or psychological distress in the populations studied. There was, however, heterogeneity in the studies reviewed, with a wide age range of participants, outcomes used and populations. There may therefore be subgroups where the association between low birth weight and depression is stronger than that seen overall. Some variables that might plausibly contribute to heterogeneity (e.g. family history of depression, smoking, maternal

physical health) were not routinely reported in most of the studies linking depression to VLBW. To conclude from this meta-analysis, data is still misleading as to link babies born between 1,000 and 1,500 g and depression.

Neurobiology behind the Insults in Utero/Being 1,000–1,500 g in Weight at Birth; the Influencers of the Adverse Outcomes

Serial head ultrasounds are a valuable bedside tool for following brain development in even the sickest preterm infants. Two studies have demonstrated that many preterm infants have a reduction in the size of the corpus callosum at term (compared with term controls); this is associated with lower gestational age at birth and with cerebral palsy and lower cognitive scores [50, 51]. Another study found that more infants with birth weight below 1,500 g who had sub-ependymal cysts noted on neonatal ultrasounds had delayed motor development at 2 years than controls with normal head ultrasounds [52]. In a thoughtful and convincing paper, Leviton et al. [53] suggest abandoning the current widely used but outmoded grading system for intraventricular haemorrhage and propose a simpler method that indicates presence or absence of subependymal haemorrhage, intraventricular haemorrhage, ventriculomegaly, and parenchymal abnormality. Although ultrasound evidence of intraparenchymal haemorrhage or white matter injury is a strong predictor, it is not diagnostic: 28–30 % of infants with these findings and birth weight below 1,000 g have no neurodevelopmental impairment at 18–22 months [54]. As many as 39 % of children with normal head ultrasounds had neurodevelopmental impairment. A number of MRI studies describe normal and abnormal brain development in preterm infants. A Swedish study of infants born before 27 weeks gestation in 2004–2005 found that 18 % had moderate to severe white matter abnormalities on head MRI at term [55].

White matter abnormalities were associated with severity of illness, use of inotropes, severe intraventricular haemorrhage and post-haemorrhagic hydrocephalus. Postnatal dexamethasone decreased total and regional cerebral tissue volumes on MRI in infants with birth weight below 1,000 g [56]. Although previous studies have shown that preterm infants with brain injury have decreased volume of both white and cortical gray matter, preterm infants with no brain injury have lower volume of white, but not gray, matter at 42 weeks postmenstrual age than full term controls [57]. A study of MRI at term in 167 infants born before 31 weeks gestation found that 21 % had moderate to severe white matter injury, and this was predictive of severe motor delay and cerebral palsy, even when controlling for ultrasound, perinatal, and neonatal risk factors [23].

A number of studies using diffusion tensor magnetic resonance imaging (DTI) to visualize white matter tracts in neonates with white matter injuries and in older children with cerebral palsy have published promising results [58–62]. One study of 24 infants with birth weight below 1,500 g who had DTI at 37 weeks postmenstrual age found a strong correlation between low fractional anisotropy values in the posterior limbs of the internal capsule and both diagnosis of cerebral palsy and severity of gait problems on outcome evaluations at 4 years [61].

Despite these promising reports, definitive predictive data that include sensitivity, specificity, and especially positive and negative predictive values for neurodevelopmental disabilities are needed before MRI or DTI become standard clinical practice, as discussed by Dammann and Leviton [63]. Neonatal illnesses, especially chronic lung disease/broncho-pulmonary dysplasia, are risk factors for neurodevelopmental impairment in preterm infants [3, 4]. A study of infants with birth weight below 1,000 g found that their logistic regression model with clinical variables better predicted neurodevelopmental impairment at 18–22 months than one with only neonatal ultrasound data; adding the ultrasound data to their model did not improve prediction [64].

Anderson and Doyle [64] demonstrated that lung disease is not only a risk factor for cerebral palsy and cognitive impairment, but also for language delay, visual-motor impairments, low average intelligence, academic difficulties, attention

and behaviour problems, memory deficits and executive dysfunction. Necrotizing enterocolitis, especially with surgical intervention, increases a preterm infant's risk for cerebral palsy, and cognitive and visual impairment [65, 66]. Trials that evaluate neurodevelopmental outcome are providing important data regarding safety and efficacy of NICU treatment strategies. Although hypotension is a risk factor, we do not know what constitutes hypotension in extremely preterm infants and whether treatment with inotropes or hydrocortisone influences neurodevelopmental outcomes [67–70]. A number of studies reporting follow-up evaluations of preterm infants enrolled in NICU randomized controlled trials provide important information as to whether rates of neurodevelopmental disability (or death or major neurodevelopmental disability) are lower (e.g. caffeine [71]), unchanged (e.g. methods of ventilation, positive airway pressure or lucinactant surfactant [72–75]) or higher (e.g. some adverse effects with inhaled nitric oxide [76, 77]). The controversy over beneficial vs. adverse effects of inhaled nitric oxide in preterm infants with lung disease can only be answered by more follow-up data from trials, especially as they vary as to selection criteria and dose administered [76–82]. An intriguing paper looked at the effect of COX2 genotype in 5.5-year old preterm children, and found lower cognitive scores with the C allele (92.7 vs. 97.6, $P<0.04$) [83]. As the effect was independent of confounding variables, the authors speculate that polymorphisms of the COX2 gene may influence the preterm brain's response to inflammation and limit the efficacy of prophylactic indomethacin on neurocognitive outcomes. This paper reminds us that preterm birth and neurodevelopmental outcome of children born preterm are the result of multiple gene–environment interactions.

Putative Developmental Mechanisms Involved

Long term prenatal and perinatal cohort studies before the introduction of neonatal intensive care concluded that social factors and the quality of the home environment can compensate for perinatal and neonatal disadvantage [84]. Recent evidence shows that favourable social and environmental factors are predictive of catch up in cognitive and behavioural development in larger LBW and preterm infants [85]. After approximately 2 years of age, IQ scores in low to moderate risk premature children are better explained by caretaking environment than their initial neonatal morbidity [86]. These findings indicate that in the vast majority of larger LBW children no persistent central nervous system insult is present. Larger preterm infants benefit from educational stimulation and home interventions [87, 88]. In contrast, although social factors are important for predicting psychological outcome in VLBW infants [89], biological factors have been found to be by far the best predictors of cognitive and behavioural outcome into school age in VPIs [17, 90–92]. Intensive intervention programmes that implemented improvements of educational stimulation and home environment have been disappointing; resulting in no long term benefits for VLBW children [93]. Taken together, this evidence suggests that VLBW children have been subject to various degrees of central nervous system insult that reduce the ability to take advantage of environmental offers. The pathogenic pathways are not fully understood but injuries to the white matter (subcortical ischaemic/infarctive brain lesions) with subsequent implications for late migration, brain organisation, and myelination are a likely cause [90, 91].

Conclusion

Although the majority of children born preterm do not develop major impairments, more preterm children than full term children develop cerebral palsy and/or cognitive impairments, and the risk increases with decreasing gestational age. The additional risk of the more subtle impairments of attention, executive function, language, visual-perceptual abilities, and fine motor function that influence the ability to function at school and at home has become apparent. While most studies focus on the most immature infants, there is growing recognition that infants born at 34–36 weeks gestation have higher mortality, morbidity, and cerebral palsy rates than fullterm infants. Mortality and neurodevelopmental outcomes of preterm infants are due to the causes of preterm birth, immature

organ systems not being up to the task of fully sustaining extrauterine life, adverse effects of obstetric and neonatal treatments, and genetic factors that we know little about. Multicentric studies using uniform methodology often note significant differences in mortality and morbidity among centres. Just as large studies of mothers who deliver preterm provide insight into the pathophysiology of preterm birth, preterm neurodevelopmental outcome studies provide insight into how the preterm brain develops and recovers from injury. Knowledge of neurodevelopmental outcome is the key to developing better treatment strategies.

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Part XI

Fetal Nephrological Development: Up to Second Trimester

Development of Renal Tissues in First Twenty Weeks

31

Rajendra Pandey

We have on average one million nephrons and they're fully formed before we're born. Premature birth and low birth weight coincides with fewer nephrons. No new nephrons are formed after birth and we lose up to 6,000 of them each year, making old age and low birth weight a risk factor for kidney disease.

The kidneys are the most intelligent organ ever evaluated in nature. They not only filter out all that is unwanted, but reclaim almost every wanted thing that escapes filtration barrier. They play a very important role in keeping skeleton stronger by participating in vitamin D synthesis. The Kidney plays a major role as an endocrine organ by secreting Erythropoietin responsible for effective erythropoiesis. The kidney by regulating RAS (Renin Angiotensin System) maintains blood pressure to make blood reach every tissue in human body.

The Urinary system serves the purpose of maintaining the electrolyte and the water balance of the body fluid that bathes the tissue. The three nephric system develop from the intermediate mesoderm located on either side of the dorsal body wall. The first of this called **Pronephros is a** small group of nephrotomes developing in the cervical region which are short lived and remain nonfunctional. They regress by fourth week and are succeeded by the **Mesonephros** developing in thoracic and lumbar region. This is comprised of simple nephrons complete in all respect, functional and draining into mesonephric (Wolffian) duct which grows caudally to open in to the primitive urogenital sinus. A pair of ureteric buds appears by fifth week, sprouting from the distal mesonephric duct and induces the overlying sacral intermediate mesoderm to develop in to **Metanephros** (definitive kidney) (*Human Embryology*, 3rd Ed, Pg. 265).

During gastrulation the mesoderm is deposited on either side of the midline and thereafter differentiates into three subdivisions: the paraxial, intermediate and lateral plate mesoderm. The intermediate mesoderm gives rise to the nephric structure of the embryo, portions of the gonads, and to the male genital duct system. Three sets of nephric structures develop from the intermediate mesoderm in cradiocaudal sequence. These are known as **cervical nephrotomes**, the **mesonephros**, and the **metanephros** (*Human Embryology*, 3rd Ed., Pg. 268).

The Cervical Nephrotomes

By early fourth week, five to seven paired cervical segments of intermediate mesoderm give rise to small but hollow ball of epithelium known as

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nephric vesicle or **nephrotome** or **pronephros**. Unlike some lower vertebrate these do not develop into the primitive functional excretory structure of a true pronephros, rather they cease developing and disappear by day 24 or 25 (*Human Embryology*, 3rd Ed., Pg. 268).

The Mesonephros

The next series of development takes place in thoracic and lumbar region (up to third lumbar level). Here by fourth week **nephric tubules** begin to develop within a pair of elongated swelling of intermediate mesoderm on either side of vertebral column. These swellings are known as **mesoneproi** or **mesonephric ridge**. About 40 mesonephric tubule develop in craniocaudal succession. While new ones are formed the older one regresses so that at one time only 30 pairs are there. By fifth week the cranial regions of mesonephros goes in to massive regression leaving only about 20 pairs of tubules occupying the first three lumbar levels (*Human Embryology*, 3rd Ed., Pg. 269).

These mesenephric tubules differentiate into excretory units that resemble an abbreviated version of the adult nephron. The **Bowman's capsule** a cup shaped sac) formed out of medial end of these tubules wraps around a bunch of capillaries called **Glomerulus** to form **renal corpuscle**.

The appearance of **mesonephric ducts** at about 24 days, a pair of solid longitudinal rods that condense in the intermediate mesoderm of the thoracic region dorsolateral to the developing mesonephric tubules. These grow caudally through the proliferation and migration of the cells at their caudal tip. With their growth into the lumbar region they diverge from the intermediate mesoderm and grow towards ventrolateral wall of cloaca to fuse with them eventually. During fusion with cloaca they begin to cavitate at their distal end to form a lumen which progresses cranially, transforming the rods into the **mesonephric duct** (*Human Embryology*, 3rd Ed, Pg. 269).

Each mesonephric tubules at its lateral tip fuses with the mesonephric duct to form a continuous passage from the excretory unit to the cloaca. These units function between 6 and 10 weeks and gradually stop functioning and then eventually regress.

The Metanephros

This is the final stage of development of definitive kidney. These start as early as fifth week. A pair of new structure known as **ureteric bud** arises out of intermediate mesoderm at the sacral region from the distal portion of the mesonephric duct (day 28). After about 4 days these ureteric bud penetrates a portion of sacral intermediate mesoderm known as metanephric blastema and starts bifurcating. As the ureteric bud branches, each new growing tip (**ampulla**) acquires a cap like aggregate of tissue from metanephric blastema, giving the metanephros a lobulated appearance. By halfway sixth week the developing metanephros consists of two lobes with a sulcus in between, but acquires 14–16 lobes by the end of 16th week. The lobular structure eventually is obscured as the sulci get filled (*Human Embryology*, 3rd Ed, Pg. 271).

The metanephric blastema and the ureteric bud exert reciprocal inductive effects. Interaction between these tissues is a classic model of induction where several hours of direct contact between ureteric bud ampulla and metanephric blastema is needed to induce differentiation in blastema tissue. Conversely, metanephric blastema through inductive signals regulates orderly branching and growth of the bifurcating tips of the ureteric buds. The collecting duct system is result of sequential bifurcation of the ureteric bud. The ureteric bud undergoes an exact sequence of bifurcations, and the expanded major and minor calyces arise through phases of intussusceptions in which occurs coalescence of earlier formed branches. Urine produced by the mature nephron flows through collecting tubules, minor calyces, major calyces, the renal pelvis and finally the ureter. In the sixth week uretric bud by four time bifurcation forms 16 branches which then coalesce to form **major calyces** extending from the renal pelvis (formed by splaying of ampulla of the ureteric bud. In the seventh week next four generation

of branches also coalesce to form **minor calyces**. By end of 32 weeks 11 additional branching takes place to form one to three million branches which will become **collecting tubules (collecting ducts)** in future (*Human Embryology*, 3rd Ed, Pg. 272–3).

The ampulla of a collecting duct is surrounded by a cap of metanephric blastema which gives rise to origin of a **Nephron** as a vesicle. As the vesicle elongates to form a tubular structure, a capillary glomerulus forms near one end of it. The tubule epithelium abutting the glomeruli thins and invaginates to form a Bowman's capsule. Bowman's capsule in union with glomerulus is named Renal corpuscle. While renal corpuscle is shaping the lengthening nephric tubules differentiates to form proximal convulated tubule, the descending and ascending limbs of loop of Henle, and the distal convulated tubules. This definitive nephron along with renal corpuscle is also called a **metanephric excretory unit**. By tenth week the distal convulated tubules unite with the collecting duct to make metanephroi functional. Now the plasma flowing through the glomeruli forms a dilute filtrate that travels through the different segments of the tubule to form concentrated urine (*Human Embryology*, 3rd Ed, Pg. 273).

The Kidney architecture gets its definite shape between 5th and 15th week. The outer cortex comprises of nephron whereas the inner medulla contains collecting ducts and loop of Henle. Each minor calyx drains a tree of collecting ducts within a **renal pyramid**. These renal pyramids are interspersed with nephron containing cortical tissue named **renal columns** of **Bertin. Renal Pyramids** converge to form the **renal papilla**. The neurons of the kidney, responsible for blood flow regulation and secretion, arise from the neural crest cells that invade the metanephron early in their development (*Human Embryology*, 3rd Ed, Pg. 274).

Kidney Diseases

Kidney diseases usually involve damage to the nephrons and can be acute or chronic. In acute kidney disease there is a sudden drop in kidney function. It is usually caused by loss of large amounts of blood or an accident and is often short lived, though it can occasionally lead to lasting kidney damage. Chronic kidney disease (CKD) is defined as loss of a third or more of kidney function for at least 3 months. In CKD kidney function worsens over a number of years and the problem often goes undetected for many years because its effects are relatively mild. Some of the symptoms associated with CKD are: headache, fatigue, high blood pressure, itching, fluid retention, shortness of breath.

However, kidney disease can lead to kidney failure (less than 10 % kidney function). Once this happens, patients need dialysis or a kidney transplant to stay alive. The risk of developing CKD is increased by old age, diabetes, high blood pressure, obesity and smoking. At least 8 % of the European population (40 million individuals) currently has a degree of CKD, putting them at risk of developing kidney failure. This figure is increasing every year and there are not enough organ donors to provide transplants for so many patients. This makes the development of new therapeutic options for treating CKD increasingly important.

Kidney Stem Cells

Scientists are still debating whether kidney stem cells exist in the adult body and if so, where they are found and how they can be identified. Cells found in a number of places within the nephrons have been proposed as candidates for kidney stem cells. The most convincing evidence for the existence of such stem cells is the discovery of a group of cells at the urinary pole of the Bowman's capsule of the nephron (marked in blue in the diagram above). These cells have some of the key features of stem cells and researchers have shown them to be responsible for production of podocytes – specialised cells involved in the filtration work of the nephron and that need to be replaced continuously throughout our lifetime. Studies also suggest that these same proposed stem cells might be able to generate a second type of specialised cell found in the nephron lining, called proximal tubular epithelial cells. Other suggested locations for kidney

stem cells include certain places in the tubules (marked green in the diagram). As well as kidney stem cells, cells with some of the characteristics of mesenchymal stem cells have very recently been isolated from the kidney.

Kidney Disease and Mesenchymal Stem Cells

A number of different types of cells from the bone marrow have been tested in animals and in clinical studies for potential use in kidney disease. Amongst all the cells under investigation, mesenchymal stem cells (MSCs) have shown the most promising results to date. Studies suggest that MSCs may be able to enhance the intrinsic ability of the kidney to repair itself.

MSCs of the bone marrow can differentiate to produce specialised bone, fat and cartilage cells. Researchers investigating the therapeutic effects of these MSCs within the kidney have suggested these cells may release proteins that can help kidney cells to grow, inhibit cell death and that could encourage the kidney's own stem cells to repair kidney damage. Further research is needed to establish whether these ideas are correct and if so, how this could lead to a treatment for patients.

Cells with some of the features of MSCs appear to exist in many other organs as well as the bone marrow, though there is much controversy amongst scientists about the exact nature of such cells and their roles in the body. Recently, cells with MSC-like features have been isolated from the kidney. These so-called kidney MSCs are distinctly different from bone marrow MSCs and heart MSCs. More research is needed to identify their precise role in normal kidney maintenance and to investigate their potential to enhance the kidney's ability to regenerate or repair itself after damage.

How Else Could Stem Cells Help Tackle Kidney Disease?

Another type of stem cell that scientists are using in kidney research is the induced pluripotent stem cell (iPSC). Induced pluripotent stem cells are made by reprogramming adult, specialised cells of the body to act like embryonic stem cells. They have the ability to develop into any cell or tissue in the body. Recently researchers have been able to use iPSCs to produce kidney cells in a very early stage of development. These very early kidney cells resemble cells found in the embryo that will turn into the cells that eventually make up the kidney in foetal development. These cells could have the potential to make the glomerulus and tubules, the building blocks of the nephron. However, a lot of research needs to be done before such cells can be used in patients to treat CKD.

An alternative approach to organ replacement is also under investigation and may help kidney disease patients in the future: The use of organ scaffolds to produce whole, transplantable organs. Organ scaffolds are organs from which all the cells have been removed. What remains is the extracellular matrix – the part of the organ that supports its shape. This matrix can be seeded with a patient's own cells, which can be carefully nurtured to grow and multiply to re-cover the scaffold. By using the patient's own cells, the complications of immune rejection that can occur with organ transplantations are drastically reduced. The challenge with this approach is identifying and obtaining the right types of cells to seed the scaffold, especially in organs with complex structures made up of many different cells. IPSCs or the recently identified kidney MSCs could be useful candidate cells for seeding kidney organ scaffolds. Very recently, experiments in rats have shown the feasibility of this approach.

Can Stem Cells Be Used to Treat Kidney Disease Today?

Stem cell treatments for kidney disease have not yet been developed. The kidney is a very complex organ consisting of a large number of different types of cells. To make a new kidney in the lab, all these different cells would need to be produced in a different way and mixed together in the hope that they would eventually recreate a

functional kidney. What's more, kidney disease comes in many flavours with different cells affected and so treatments aiming to replace damaged cells within a patient's kidney would need to supply different types of cells for different patients. Research on organ or cell replacement therapies is ongoing, but this is likely to be a long-term goal.

In the meantime, stem cells may benefit patients in other ways. For example, stem cells can be used to help progress our understanding of the disease through studies on the development and behaviour of kidney cells grown in large numbers in the laboratory. Stem cell research may also enable us to utilise the body's own repair mechanisms to find treatments for kidney disease. In acute kidney disease, the body can often repair kidney damage itself, but it is unable to do this well enough to tackle the progressive damage that occurs in chronic kidney disease. The recent identification of mesenchymal-stem-cell-like cells in the kidney may open up new possibilities for enhancing the body's own capacity for regeneration and repair of damaged kidneys. Investigating these possibilities by studying how these newly discovered cells work is currently an important area of research. Researchers also continue to explore new ideas using emerging technologies in stem cell research, such as reprogramming cells to change their behaviour.

1. Congenital anomalies of the kidney and urinary tract (CAKUT) constitute approximately 20–30 % of all anomalies identified in the prenatal period [1].

Defects can be bilateral or unilateral, and different defects often coexist in an individual child. The antenatal screening and postnatal evaluation of infants with CAKUT are discussed in greater detail separately. (See "Evaluation of congenital anomalies of the kidney and urinary tract (CAKUT)" and "Congenital ureteropelvic junction obstruction" and "Primary megaureter in infants and children" and "Ectopic ureter" and "Renal ectopic and fusion anomalies" and "Autosomal recessive polycystic kidney disease in children". Congenital abnormalities of the kidney and urinary tract are frequently observed in children and represent a significant cause of morbidity and mortality. These conditions are phenotypically variable, often affecting several segments of the urinary tract simultaneously, making clinical classification and diagnosis difficult. Renal agenesis/hypoplasia and dysplasia account for a significant portion of these anomalies, and a genetic contribution to its cause is being increasingly recognized. Nevertheless, overlap between diseases and challenges in clinical diagnosis complicate studies attempting to discover new genes underlying this anomaly. Most of the insights in kidney development derive from studies in mouse models or from rare, syndromic forms of human developmental disorders of the kidney and urinary tract. The genes implicated have been shown to regulate the reciprocal induction between the ureteric bud and the metanephric mesenchyme. Strategies to find genes causing renal agenesis/hypoplasia and dysplasia vary depending on the characteristics of the study population available. Congenital abnormalities of the kidney and urinary tract are frequently observed in children and represent a significant cause of morbidity and mortality. These conditions are phenotypically variable, often affecting several segments of the urinary tract simultaneously, making clinical classification and diagnosis difficult. Renal agenesis/hypoplasia and dysplasia account for a significant portion of these anomalies, and a genetic contribution to its cause is being increasingly recognized. Nevertheless, overlap between diseases and challenges in clinical diagnosis complicate studies attempting to discover new genes underlying this anomaly. Most of the insights in kidney development derive from studies in mouse models or from rare, syndromic forms of human developmental disorders of the kidney and urinary tract. The genes implicated have been shown to regulate the reciprocal induction between the ureteric bud and the metanephric mesenchyme. Strategies to find genes causing renal agenesis/hypoplasia and dysplasia vary depending on the characteristics of the study population available [2]. Abnormal genes cause polycystic kidney dis-

ease, and the genetic defects mean the disease runs in families.

There are two types of polycystic kidney disease, caused by different genetic flaws:

- **Autosomal dominant polycystic kidney disease (ADPKD)**. Signs and symptoms of ADPKD often develop between the ages of 30 and 40. In the past, this type was called adult polycystic kidney disease, but children can develop the disorder.
  Only one parent needs to have the disease in order for it to pass along to the children. If one parent has ADPKD, each child has a 50 % chance of getting the disease. This form accounts for about 90 % of cases of polycystic kidney disease.
- **Autosomal recessive polycystic kidney disease (ARPKD)**. This type is far less common than is ADPKD. The signs and symptoms often appear shortly after birth. Sometimes, symptoms don't appear until later in childhood or during adolescence.
  Both parents must have abnormal genes to pass on this form of the disease. If both parents carry a gene for this disorder, each child has a 25 % chance of getting the disease.

Researchers have identified two genes associated with ADPKD and one associated with ARPKD.

In some cases, a person with ADPKD has no known family history of the disease. However, it's possible that someone in the affected person's family actually did have the disease, but didn't show signs or symptoms before dying of other causes.

In a smaller percentage of cases where no family history is present, ADPKD results from a spontaneous gene mutation.

Acknowledgement

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Part XII

Fetal Haematological Development

Development of the Haemopoiesis System before the Second Trimester of Pregnancy

32

Mainuddin Naskar and Niranjan Bhattacharya

Introduction

During embryogenesis the extra–embryonic yolk sac, fetal liver, preterm bone marrow and developing placenta are spatially and temporarily distinct sites where haemopoiesis [1] occurs. Soon after the implantation of blastocyst, erythropoiesis is established and primitive erythroid cells appear in the Yolk Sac Blood Islands by day 18th of gestation [2].

The origin of haemopoietic cells is closely related with gastrulation and formation of mesoderm. Inducers of mesoderm are Transforming Growth Factor b (TGF-b), Fibroblast Growth Factor (FGF), Bone Morphogenic Protein-4 (BMP-4). These factors play an important role in haemopoiesis. Yolk sac erythroblast develops in close association with first embryonic blood vessel-which suggests that blood cells and endothelial cells are of common haemangioblast precursor's source.

In Yolk sac haemopoiesis the yolk sac erythroblasts have several characteristic features thereby distinguishing them from their later counterparts. Primitive nucleated erythroblast differentiates within vascular network rather than extravascular space and in circulation.

Primitive erythroblasts are characterized by:

1. More rapid maturation.
2. Increased sensitivity to erythropoietin.
3. Shortened life span compared to fetal and adult erythroblast.

Yolk sac erythroblasts are extremely large red cells with mean cell volume (MCV) of 450 fl/cell. The erythroid progenitors burst forming-erythroid (BRU-E), and Colony forming unit-erythroid (CFU-E) appear in yolk sac at fourth week of gestation. Primitive erythroblast and erythroid progenitors then enter the embryo proper through the circulation. After 7 weeks, haemopoietic progenitors are no longer detected in the yolk sac. The primitive erythroblasts derived from yolk sac continue to circulate up to 12 weeks of gestation [3].

From 9th to 24th weeks of gestation the liver serves as a primary source of red cells. Between 7th and 15th week of gestation, 60 % of liver cells are haemopoietic. The fetal-liver derived definitive macrocytes are smaller than yolk sac megaloblasts and contain one third the amount of

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haemoglobin. Differentiation of erythroid cells in fetal liver is dependent on erythropoietin signaling through its receptor and the JAK2 kinase. In the liver, erythropoietin transcripts are present during the first trimester of pregnancy. The liver remains the primary site of erythropoietin transcription throughout the fetal life. In 17 weeks of gestation erythropoietin transcripts also remain present in fetal kidney and it increases after 30 weeks. Erythropoietin is expressed both in fetal liver and postnatal kidney. Like primitive erythropoiesis in the yolk sac, definitive erythropoiesis in the fetal liver is essential for continued survival of the embryo [4].

In yolk sac haemopoiesis is restricted to erythroid and macrophage cells but haemopoiesis in fetal liver includes other myeloid and lymphoid lineages. Megakaryocytes are present in the liver at 6 weeks of gestation. Platelets are first evident in the circulation at 8–9 weeks of gestation. Small numbers of circulating leukocytes are present in the 11th week of gestation. Granulopoiesis is present in liver parenchyma and in some areas of connective tissue as early as 7 weeks of gestation. Despite the low number and immature appearance of hepatic neutrophil, the fetal liver contains haemopoietic progenitor cells such as colony-forming unit-granulocyte-monocyte-macrophage (CFU-GEMM) and colony-forming unit-granulocyte-monocyte (CFU-GM). CFU-GM growth depends upon several cytokines, including granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), and interleukins [4, 5].

In marrow haemopoiesis, haemopoietic cells are first seen in the marrow of 10–11 week's embryo, [1, 2] and they remain confined to the diaphyseal region of long bones till 15 weeks of gestation.

Initially there is equal number of myeloid and erythroid cells in the fetal marrow. However, by 12 weeks of gestation myeloid cells predominate and the myeloid to erythroid ratio approaches to adult level of 3–1 by 21 weeks of pregnancy. Macrophage cells in the fetal marrow express the lipopolysaccharide receptor CD14. The marrow becomes the major site of haemopoiesis after the 24th week of gestation [6, 7].

Lymphopoiesis is present in lymph plexus and the thymus beginning at 9 weeks of gestation. B cells with surface IgM are present in liver and circulating lymphocytes at 9 weeks of gestation. Lymphocyte subpopulation are detected in fetal liver at 13 weeks of gestation [8]. Absolute numbers of major lymphoid subsets in 20–26 weeks old fetuses defined by antigens CD2, CD3, CD4, CD8 CD19, CD20 and CD16, are similar to those of the newborn [9, 10].

The reconstitution of haemopoiesis by cord blood transplantation indicates that hematopoietic stem cells are present at birth. The immunological reconstitution of an immunodeficient human fetus with fetal liver derived cells indicates that haemopoietic stem cells are present in the fetal liver. Yolk sac stem cells were first thought to seed the liver and eventually the bone marrow. The transient appearance of CD34-positive blood cells associated with ventral wall of the aorta in a 5 week human embryo suggests that the AGM-region-derived stem cell seed the liver and the marrow to provide lifelong haemopoiesis [11].

Human hemoglobin is a tetramer composed of two A-type and two B-type globin chains. The A-globin gene cluster is located on chromosome no. 16 [12] and the B-globin gene cluster is located on chromosome no. 11 [12]. During embryogenesis the genes of both chromosomes are activated sequentially [13].

Hb Gower 1 is the major hemoglobin in an embryo less than 5 weeks of gestation. Hb Gower 2 has been found in embryos of gestational age as low as 4 weeks and absent in embryos older than 13 weeks [14, 15].

A newborn represents the culmination of developmental evidence from conception and implantation through organogenesis. The embryo requires red cells for transport of maternal oxygen to permit the growth and development. Embryonic haemopoiesis begins with the generation of primitive RBC which supplies embryos with immediate oxygen demand. In the placental villi of first trimester, maturation and enucleation of human primitive RBC occurs. Frequency of enucleated RBC is found to be higher in placental villous stroma than circulating erythrocytes during 5–7 weeks of embryonic development. Macrophage progenitors of fetal origin present in the chorionic plate of the placenta before fetoplacental circulation, and migration of macrophage to villous of

placenta after the fetoplacental circulation, proves that the macrophage has its role in fetal haemopoiesis and enucleation of RBC. Birth brings dramatic changes in circulation and oxygenation which affects haemopoiesis as the newborn makes a separate biological existence [16, 17].

Hb F is the major hemoglobin of fetal life [18]. Synthesis of HbA can be demonstrated in fetuses as young as 9 weeks of gestation [19, 20]. In fetuses of 9–21 weeks of gestation the amount of Hb A rises from 4 to 13 % of the total hemoglobin. This level of Hb A enables the antenatal diagnosis of b Thalassemia using globin chain synthesis.

The fetal hemoglobin or HbF concentration in blood decreases after birth by about 3 % per week and comprises less than 2–3 % of total hemoglobin at 6 month of age. This rate of decrease is related to gestational age and not to the environment or oxygen tension that occurs at the time of birth.

The fetal blood composition changes markedly during the second and third trimesters. The mean hemoglobin in fetuses progressively increases from 9.0 ± 2.8 g/dl at 10 weeks to 16.5 ± 4.0 g/dl at 39 weeks.

There is decrease in the MCV fetal red cells from a mean of 134 fl/cell at 18 weeks to 118 fl/cell at 30 weeks of gestation.

The total white blood cell count during the middle trimester is between 4 and 4.59 × 109/l with an 80–85 % preponderance of lymphocytes and 5–10 % neutrophils. The circulating nucleated red cell decreases from a mean of 12 % at 18 weeks to 4 % at 30 weeks. The platelet count remains greater than 15,000/μ ml from 15 weeks gestation to term [21, 22].

Large numbers of haemopoietic progenitors circulate in fetal blood. Blood samples obtained by fetoscopy at 12–19 weeks of gestation reveals a mean of 20,450 BFU-E/ml and 12,490 CF-GM/ml [23].

Placenta is the site for feto-maternal exchange where nutrients, oxygen, metabolic by-products, hormones and many other molecules pass from fetus to maternal circulation. As per concentration of many molecules in maternal circulation placenta transfers them in fetomaternal pool. Maternal blood flow, fetoplacental blood flow and placental trophoblastic [23] membrane permeability are the main modifiers of placental transfer function. Fetal oxygenation, nutrition and metabolism are modified by

1. Altered maternal perfusion
2. Altered placental perfusion
3. Reduction of placental permeability
4. Increase in placental metabolic requirement.

Fetomaternal transfer through placenta may be through diffusion, or be a carrier mediated transfer or active transfer. All are dependent upon placental properties such as fetomaternal blood flow, pattern of perfusion through placenta, surface thickness and property of placental membrane and the metabolic activity of the placenta.

Optimal fetal oxygenation is assured by:

1. High uterine blood flow.
2. Production of fetal hemoglobin in sufficient amount which has greater oxygen affinity than maternal hemoglobin.
3. Uniform fetal perfusion of placenta.
4. Increased tissue perfusion.
5. High fetal cardiac activity.

For embryonal growth, fetal growth and metabolic requirement, normal placental development and maximizing placental efficacy are essential [24].

For flourishing intrauterine pregnancy, the following are essential

(a) Trophoblast to anchor and invade the deciduas.
(b) Ability of uterine vasculature for progressive dramatic increase of blood flow.

After implantation a remodeling of uterine epithelium and stroma occurs and a line of nutrient develops from the maternal tissue to the growing fetus. Endometrial invasion occurs by:

(a) Interstitial invasion of trophoblast in decidua and stroma.
(b) Endovascular invasion by which vascular remodeling is initiated.

Both infiltrative and phagocytic components are involved in interstitial invasion of the endometrium

by the conceptus. The erosive procedure is indicated by far more molecular signals [25] and there may be a linkage with primitive immune mechanisms; there is a presence of adequate killer cells in the late luteal phase and early pregnant human epithelium. Retrograde trophoblast extension in spiral vessels begins in venous circuit and its flow is against the arterial tree [26]. Remodeling of trophoblast is a continuous process and occurs in two sequences. The first sequence is completed in late first trimester and the second sequence of remodeling is completed by early second trimester. After the second sequence, the trophoblast penetrates the superficial layer of the myometrium.

Uterine artery flow in non-pregnant state is 1–2 % of cardiac output and this increases to 30–50 % of cardiac output at 20–24 weeks of gestation. Non-pregnant spiral artery flow is high resistance and low volume whereas spiral vessels of remodeling trophoblast are low resistance and high capacity.

There is comparative and temporary denervation of uterine artery vasculature in pregnancy [26, 27].

Uteroplacental and fetoplacental circulation should depend on anatomic and humoral mediator. Trophoblastic invasion is less in the periphery of the placenta than the center [28] and thus less involved in fetomaternal exchange.

Anatomically there are three types of trophoblast:

1. Villous trophoblast lining the intervillous space with cytrotrophoblast "stem cell" and thus producing syncytiotrophoblast.
2. Anchoring mononuclear trophoblast attached to basal plate.
3. Invasive, Interstitial and endovascular trophoblast.

All types of trophoblast differentiate and express antigen in highly coordinated and distinct pattern that are influenced by soluble factors and extracellular matrix components [29, 30]. Invasive trophoblast expresses human leucocyte antigen G (HLA-G), a major histocompatibility antigen, expression of which is restricted to a few cell types, including trophoblast [29]. Implantation and early placental development occurs under great regulation and modulation of trophoblastic function.

Placental functional maturation occurs by villous morphological changes in pregnancy. The primary villi are columns of cytotrophoblast. "Secondary villi" develops after mesodermal invasion in the primary villi. The appearance of blood vessels in secondary villi transforms them into tertiary villi. The mature villous functional unit is a barrel, with the staves formed by the large fetal stem vessels and the villous tree arborizing towards the center where recently formed villi are found.

By the end of the fifth week of gestation the embryonic heart begins to pump nucleated erythrocytes from the yolk sac throughout villous circulation. There is a good co-relation between the proportion of nucleated and anucleated RBC and crown- rump length of the fetus [31]. Villous macrophages (Hofbauer cells) are numerous throughout gestation (about 40 % of stromal cells); near term its number becomes less.

Villi develop over the entire conceptus but atrophy over extra placental membranes occurs by the end of the first trimester. Normal villous atrophy may be confused with placental infarct. Remnants of villi in placental membrane are chorionic lave and the villi developed in the placental disc are chorion frondosum.

The delivered placenta is deflated compared to the uterus. The average diameter of the placenta is 18.5 cm (range 10.5–24.5 cm) with mean thickness of 2.3 cm (range 1.1–4.1 cm).

Early stage growth of placenta is due to growth of chorionic disc whereas late stage of gestation placental weight gain is due to increase in placental thickness.

The mean diameter of the placenta at the third to sixth month is 5.8 cm, 8.2 cm, 10.8 cm and 13.0 cm respectively. The number of major villous trunks remain the same. Ultrasonic data review of the mean weekly increase of placental volume between 16 and 24 weeks is 31 ± 8 cm.

This is in a nutshell the development of the hemopoiesis system before the second trimester of pregnancy.

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Part XIII

Pharmacological Implication of Fetal Development: Up to Second Trimester

Liabilities of the Fetus as a Patient

33

Mursheed Ali and Subhas Chakraborty

Issues Related to Fetal Development

Twin-Twin Transfusion Syndrome

Twin-twin transfusion syndrome affects roughly 15 % of monochorionic twin pregnancies and regardless of contemporary obstetric and neonatal treatment procedures, is connected with 30–50 % perinatal mortality [1–3]. The haemodynamic changes in twin-twin transfusion syndrome are because of uneven incessant intrafetal transfusion. It is important to note that often neurodevelopmental result is poor in surviving newborn children which is inferable from complexities of the ailment itself and the high rate of preterm birth that goes with this condition [4]. The old criteria of hemoglobin discrepancy and birth weight have been replaced by a ultrasound stage based classification [5]. Overall rates of perinatal survival have increased as a consequence of a number of treatment modalities, including amnioreduction, septostomy, and laser ablation. Amnioreduction offers promising results if it is done at the right stage of the disease, with at least one fetus surviving in more than 85 % of cases and two surviving in 66.7 % of cases at stage I or stage II disease [6]. The general survival rate, according to a study, for cases before 28 weeks that were treated by laser was 58 %; similarly, amnioreduction offers good results in early stage disease, with at least one fetus surviving in more than 85 % of cases and two surviving in 66.7 % of cases with stage I or stage II disease [7–9]. Laser treatment enhances the degree of single survivor, by decreasing the number of double survivors and double deaths. The key consideration is to oversee pregnancies affected by twin-twin transfusion syndrome with early referral to a tertiary fetal solution unit with experienced practitioners. These patients need cautious assessment before any mediation, which often has to be individualized.

Selective termination of pregnancy in serious twin-twin transfusion syndrome is one alternative that is done in only a handful of centres.

Multifetal Pregnancy Reduction

Fetal reduction can be either specific or non-specific. In specific fetal decrease, one of the fetuses may have an anomaly that may be lethal and its proximity may jeopardise the survival/growth of its cotwin(s). Non-selective fetal reduction is generally done early in gestation in high order multiple pregnancies to lessen the probability of high order births with all their

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complications. Fetal reduction has the double goal of preventing the birth of a baby that may have significant abnormalities and in addition avoiding the risk of preterm delivery that is regularly connected with multiple pregnancy. Contingent upon chorionicity, multifetal pregnancy decrease could be achieved by ultrasound guided intracardiac potassium infusion, bipolar cord coagulation, or interstitial laser. In high order multiple pregnancies (triplets or more) multifetal pregnancy reduction has a certain advantages in terms of perinatal result, which is seen in lessened risks for prematurity, cerebral paralsy, and pregnancy related complications. Unless there is a discordance between the fetuses for an anomaly that caries a serious risk of handicap, most fetal terminations in the United Kingdom are done before 24 weeks of gestation, primarily between 11 and 14 weeks. One reason for choosing this period is because it is harder to do transabdominal procedures before 10 weeks because of the little fetal size and the inaccessibility of the fetuses when the uterus essesntially remains a pelvic organ. Moreover, before this time, the spontaneous loss of a fetus may happen. According to one study, multifetal pregnancy reduction is normally done between 11 and 14 weeks, principally in view of a lower miscarriage rate (5.4 %) compared with the risk of spontaneous miscarriage (12 %).

The transabdominal procedure has supplanted the transvaginal technique [10]. As the perinatal result of fewer twins approaches, yet does not reach, that of spontaneous twins, the decrease of higher order multiple pregnancies to a number of two is currently standard practice, as many groups feel that the perinatal mortality and morbidity of twin pregnancies are within acceptable limits. The Human Fertilisation and Embryological Authority has now decreed that at most two fetuses can be replaced at one time, to reduce the risks connected with high order multiples. According to a recent study of the International Registry, in 3513 patients before 24 weeks' gestation undergoing multifetal pregnancy reduction in 11 centres, the overall loss of pregnancy was 9.6 %, with 3.7 % preterm deliveries between 25 and 28 weeks of gestation [10], both of which appear better than the published results for unreduced numerous pregnancies [11]. There is a strong correlation between the starting number of fetuses and the completing number after multifetal pregnancy reduction, with the probability of poor pregnancy result (loss and prematurity) increasing with higher order multiples [12].

Fetal Red Cell and Platelet Alloimmunisation

Fetal Rh or Kell status can now be determined non-intrusively by utilizing coursing fetal DNA in maternal plasma. This mechanical development technological advancement has rendered early intrusive testing redundant in alloimmunised pregnancies. Although in utero transfusions remain the preferred treatment for iron deficient babies affected by red cell alloimmunisation, techniques for monitoring the at risk fetus have developed. Previously, serial amniocentesis was needed to measure optical density 450 levels for calculating the time of the next transfusion. Every amniocentesis carried a risk of miscarriage or preterm labour, and as a few pregnancies required many of these procedures, the risk was considerable. The approach of Doppler velocity assessment in the fetal middle cerebral artery has revolutionized the management of fetal anemia, whatever the cause. This strategy has 100 % sensitivity with a 12 % false positive rate and permits accurate timing of intrauterine transfusions without any requirement for extra invasive procedures [13]. Feto-maternal alloimmune thrombocytopenia is caused by human platelet antigen incompatibility. Fetal intracranial hemorrhages can happen in 10–20 % of cases. The accessible treatment alternatives incorporate maternal treatment with high dosage intravenous immunoglobulin, corticosteroids, a blend of both, or serial intrauterine platelet transfusions. A recent European study on the antenatal administration of feto-maternal alloimmune thrombocytopenia noted that the start of treatment can now be stratified on the basis of sibling history and supports the utilization of first line maternal immunoglobulin treatment, in this way staying away from various intrusive procedures [14].

Screening of Fetus- Prenatal Stage

As more than 90 % of auxiliary and chromosomal abnormalities emerge in pregnancies without any risk factors, anomaly and aneuploidy screening is offered universally. In a few circumstances screening for particular genetic issues may be restricted to certain ethnic groups. In case of Down's syndrome, despite older women being at higher risk, even women about 35 years are screened with this syndrome [15]. In England and Wales, prenatal diagnosis of Down's syndrome cases increased from 28 % in 1989 to 53 % in 1999, and screening programs for Down syndrome that were based on maternal serum biochemistry or ultrasound were more effective and efficient than the screening programs that used advanced maternal age alone [16]. By April 2007 the NHS is required to provide a test that has a detection rate over 75 % and a false positive rate of less than 3 %. Only the consolidated, integrated, quadruple, and serum integrated tests will meet this more stringent criteria.

Diagnostic Testing for Fetal Abnormalities

Recent advancements in fluorescence in situ hybridization and quantitative fluorescence polymerase chain reaction methods have prompted quick reporting times (1–3 days) for Down's syndrome identification of other trisomies. The quick testing of prenatal samples has brought up the issue of whether full karyotypic examination and reporting should be done for these samples. Most women who experience invasive testing do so due to the fact that they have been distinguished as being at high risk by a specific screening method. Full karyotypic examination may detect abnormalities of unknown significance (small "marker" chromosomes, balanced chromosome rearrangements, or regions of variability), which may be inherited. These findings of genetic abnormalities frequently raise ethical issues for the mothers to choose termination of pregnancy or continue with it and remain anxious for the pregnancy. Generally, 0.07–0.14 % of pregnancies which are karyotyped will have a clinically significant chromosomal abnormality that would not be detected through rapid testing [17, 18]. Preimplantation genetic diagnosis is now recognized as a reliable for diagnozing chromosomal abnormalities arising from parental balanced translocations or rearrangements [19, 20]. This procedure can be utilized to screen fetuses of in vitro fertilization pregnancies. It is expensive and intrusive, but it is suitable for women at particularly high risk because of chromosome rearrangement or those had in vitro fertilization.

Non-invasive Prenatal Diagnosis Strategies

As all current screening techniques include an invasive diagnosis test (for instance, amniocentesis or chorionic villous sampling), which carries a small but definite risk of miscarriage, endeavors have been made to develop less invasive diagnostic strategies that examine the fetal genome through isolation and characterization of DNA from fetal cells distinguished in the maternal blood circulation or investigation of free fetal DNA in maternal plasma. Circulating fetal nucleated red blood cells, mesenchymal stem cells, and trophoblast have all been utilized for different prenatal diagnostic tests [21–23]. The limitation of this technique is the rarity of such cells in the maternal circulation and further, the availability of a reliable fetal marker. The number of fetal cells in maternal circulation vary according to stages of gestation and methods employed for analysis; circulating fetal cells are rare and is estimated to be around one fetal cell in 10^4 to one in 10^9 maternal cells in normal pregnancy [24]. As the duration of pregnancy increases, the free fetal DNA increases to around 3–6 % of total DNA in maternal plasma. The fetal DNA concentrations increases during aneuploidy pregnancies [25]. Another new technique of investigating is the analysis of plasma RNA which is employed for non-invasive profiling of gene expression [26].

Recent Imaging Techniques- Fetal Magnetic Resonance Imaging

High resolution ultrasonography allows for the detection of anomalies, but poor views because of maternal obesity or oligohydramnions are limitations. There are faster imaging techniques that allow a single slice to be obtained in less than 400 ms, which obviates most fetal motion artefacts. No harmful impact on the developing fetus have yet been reported. Moreover, the fetal magnetic resonance imaging is useful for assessing abnormalities of the CNS. Levine et al. in 1997 reported that fetal magnetic resonance imaging provided additional information on abnormalities in the brain in 55 % of fetuses [27]. Another advantage of this technique is it its evaluation of changes in the developing brain due to neuronal migration, gyral formation, and myelination. MRI helps in the evaluation of other fetal anomalies like sacrococcygeal teratoma, diaphragmatic hernia, and spinal anomalies. Image acquisition time is expected to decline further and this will enhance the clear visualization of almost any fetal organs.

Fetus Examination- Three Dimensional Ultrasonography

Three dimensional ultrasonography is a moderately new strategy for examination. For a while it appeared to give only aesthetic images with little clinical value. In some clinical and research settings, three dimensional ultrasonography has been demonstrated to be advantageous in assessing the fetus [28, 29]. The indications for three dimensional ultrasound are not yet clear. Most reports have dealt with the identification of abnormalities of the fetal surface, especially cleft lip and palate and spina bifida. Very few studies have looked at the capacity of three dimensional ultrasonography to survey more profound structures. Also, three dimensional ultrasonography permits precise estimation of the volume of any fetal organ. Very few studies have investigated the clinical utility of fetal volume estimations. Once more, as this technology advances and real time three dimensional images enhance, it may become the imaging modality of choice.

Current Tests for Fetal Abnormalities

Fetal viability scans: This is an ultrasound examination done at 6–10 weeks of gestation with an aim to focus the number of fetuses present, and whether the pregnancy is advancing typically inside the uterus.

First trimester screening for fetal aneuploidies: This incorporates maternal blood tests and ultrasound evaluation of the fetus. The most effective screening test in the first trimester uses a combination of biochemical markers, pregnancy-associated plasma protein A (PAPP-A) and human chorionic gonadotropin (hCG), and the nuchal translucency (NT) measurement to adjust a woman's risk for trisomies 21 and 18. Collection of blood for biochemical analysis is performed between 9 and 13 6/7 weeks' gestation and Ultrasound assessment of the NT measurement is performed between 11 and 13 6/7 weeks [30]. Doppler ultrasound assessment of the uterine arteries is more effective in identifying preeclampsia [30].

Fetal anomaly scans: Done at 18–23 weeks of development of fetus. Definite fetal anatomy and structural abnormalities can be analyzed.

Risk reassessment scan: This scan focuses on markers and heart abnormalities of Down's syndrome (trisomy 21). This scan is attempted when a serum biochemistry test risk is high.

Cervical screening: A transvaginal ultrasound gives an image of the cervix; if it shorter than normal or there is funneling, there is possibility of preeclampsia and preterm birth.

Fetal echocardiography: Fetal echocardiography (echoCG) is considered to be an accurate diagnostic tool, reflecting postnatal outcomes well. Fetal echoCG is now widely used in pediatric cardiology and perinatology and even for fetal cardiac intervention, improving the preoperative condition, morbidity and mortality of patients with congenital heart

disease (CHD) [31]. Definite assessment of fetal heart is carried-out by a fetal cardiologist.

Fetal scan: Done at 24–40 weeks growth of fetus. The scan expects to:

1. Assess fetal head size, abdomen and fetal thigh bone for general estimation of fetal weight.
2. Assessment of placenta position and appearance.
3. Examination of fetal developments.
4. Estimation of the amount of amniotic fluid.
5. Evaluation of blood flow to the fetus and placenta through color Doppler.

Invasive Strategies

Chorionic villus sampling (CVS): Helpful for diagnosing fetal chromosomal and genetic disorders. CVS is a diagnostic procedure which involves removing some chorionic villi cells from the placenta at the point where it attaches to the uterine wall. This is usually done at 11–14 weeks of gestation [32].

Amniocentesis: Similar to CVS, but done utilizing amniotic fluid which contain fetal tissues. Both invasive methods convey risk of 1 % miscarriage.

Fetal MRI: It is a non-invasive, safe methodology executed as supplementary to ultrasound, and is valuable in fetal visceral and soft-tissue depiction like central nervous system abnormalities. MRI provides multiplanar imaging as well a large field of view, facilitating examination of fetuses with large or complex anomalies, and visualization of the lesion within the context of the entire fetal body. Fetal MRI may detect subtle neural tube defects not shown by US and determine the level of the defect in myelomeningocele for potential fetal surgery. However, it is prudent to wait until 17–18th weeks of gestation before performing fetal MRI because of the potential risk to the developing fetus and the current limitations of fetal MRI created by the small size and excessive motion of younger fetuses [33].

Fetal intrauterine transfusion: Intrauterine fetal blood transfusion (IUT) is thought to be the only life saving therapy, and very effective in the management of preterm Rh isoimmunized pregnancies. Universal use of prophylactic Rh(D) immune globulin has reduced the need for IUT dramatically; however, the procedure continues to be an essential modality for treatment of severe fetal anemia from a variety of causes, such as non-Rh(D) alloimmunization, parvovirus B19 infection, chronic fetomaternal hemorrhage, and homozygous alpha-thalassemia. The procedure is generally limited to pregnancies between 18 and 35 weeks of gestation because before 18 weeks the small size of the relevant anatomic structures causes technical challenges and after 35 weeks IUT is considered riskier than delivery followed by postnatal transfusion therapy [34].

Recent Advancements in Fetal Medicine

Fetal medicine involves the assessment of fetal health, development and wellbeing and therefore methods of fetal intervention to correct aberrations prior to birth. It requires a multidisciplinary approach involving obstetricians, maternal medicine specialists, neonatologists, paediatric cardiologists, paediatric surgical specialists, geneticists and specialist nursing counselors. Fetal medicine addresses the unborn patient. The advances made in fetal medicine now allows for prenatal screening in early first-trimester for aneuploidy fetuses and for forecast of preeclampsia. This has enhanced the detection rate to 95 % and 80 % with a false-positive rate of 2 % and 10 %, respectively [35].

Recent improvements in fluorescence in-situ hybridization or quantitative fluorescence PCR have prompted quick results within 2–6 h of amniocentesis for trisomy 21 [36]. Preimplantation genetic testing is a technique used to identify genetic defects in embryos created through in vitro fertilization (IVF) before pregnancy. Preimplantation genetic *diagnosis* (PGD) refers specifically to when one or both

genetic parents has a known genetic abnormality and testing is performed on an embryo to determine if it also carries a genetic abnormality. In contrast, preimplantation genetic *screening* (PGS) refers to techniques where embryos from presumed chromosomally normal genetic parents are screened for aneuploidy [37]. Advance in imaging with introduction of fetal MRI assesses CNS and other anomalies in the fetus [48]. Three- and four dimensional ultrasonography gives added data to investigations of skeletal deformities, facial abnormalities and evaluation of neural tube defects [38]. This technology now offers new opportunities to study embryonic and fetal growth which would make information available on specific movement patterns and quality of movement in the high-risk fetus for detection of fetal neuro-developmental impairment [39].

Until recently, the main inquiry raised by the prenatal diagnosis of a fetal abnormality was whether to abort the fetus or to wait for delivery. Presently, treatment of a few fetal disorders has turned out to be achievable in-utero. With the recent advancement in negligibly invasive fetal surgery, frequency of open hysterotomy has diminished. The latter is performed for conditions, for example, myelomeningocele, large congenital cystic adenomatiod malformation of lungs or a large sacrococcygeal teratoma [40]. Prenatal repair of fetal myelomeningocele is presently at present under scrutiny through a an appropriate randomized trial supported by National Institute of Child Health and Human Development [49]. Introduction of minimally intrusive technique for laser photocoagulation for the treatment of twin-twin transfusion syndrome [41], ablation of posterior urethral valves causing bladder outlet obstruction [42] and tracheal occlusion using a balloon catheter for congenital diaphragmatic hernia [43] are the latest fetal mediation being done in-utero. Non-intrusive prenatal diagnosis of fetal RhD status [44] and fetal anaemia has turned into a reality [45]. Then again, appraisal of fetal growth and subsequent monitoring of fetal oxygenation status can be effectively performed at the level of the fetal and umbilical vessels and haemodynamic changes can be watched and utilized for improving the characteristics of management [46]. With these advances, incorporation of the information of genomics, proteomics, and stem cell exploration and gene therapy into fetal medicine is now required so as to make a wider range of therapeutic interventions possible [47]. Further, the field needs to move into the next phase, that is, evidence-based practice of fetal medicine and surgery where it is essential not to introduce exciting techniques without solid evidence, which eventually would require establishment of and collaborations between multicentre networks of excellence globally; this would help organize and set the agenda for good quality research and clinical progress in this field.

Conclusions

Despite the fact that the diagnosis of numerous fetal anomalies can be done in-utero, the dilemma regarding what is the most suitable treatment remains. Open fetal surgery is technically possible for some of these conditions. Minimally invasive techniques and enhanced imaging coupled with novel methods to anticipate preterm labor will presumably provide more scope for therapeutic mediations. Rapid developments in genomics, proteomics and stem cell research will likewise make in-utero treatment of some genetic conditions possible. The future of fetal medicine lies in the application of basic science to advances in technology and medical science like stem cell therapy and gene therapy to further ensure normal fetal development.

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34 Fetus as a Patient During the First and Second Trimesters of Growth and Development

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Introduction

Fetus as a patient is a new concept which is starting to get more prominence in the field of obstetrics and gynecology along with other fields of medicine like pediatric cardiology, pediatric neurology and surgery and even among basic biomedical scientists [1]. Fetal medicine depends on the well being of the in utero fetus during the time of pregnancy and it mainly focuses on the different aspects of repairing and healing various fetal abnormalities which might appear during the whole duration of pregnancy. Previously treatment was restricted to the neonatal periods but with recent advancements in the basic science of medicine and surgery there is a high possibility to detect and treat any anomaly of the fetus prenatally [2].

Diagnosis of Fetal Abnormalities by Invasive and Non-invasive Techniques

Currents advancements in diagnostic tools and techniques like fetal viability scan an ultrasound examination which can determine the number of fetuses present between 6 and 10 weeks time along with detection of fetal aneuploidies by maternal blood test and a special ultrasound technique to assess the fetus for any chromosomal abnormalities particularly for Down's syndrome can be conducted [2]. In the second trimester fetal anomaly scans can be performed by assessing the detailed anatomical and physiological structures of the fetus. Also a type of risk assessment scan can be used for examining soft markers and cardiac

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anomalies and can be replaced with a serum biochemistry test in case if there is too much risk for the fetus. Fetal echocardiography tests can be also carried out for detailed evaluations of fetal cardiac anomalies [2]. Fetal well being scan is another important and latest technology by which the fetal abdominal size, head size, fetal weight, placental position, fetal movements and the volume of amniotic fluid can be assessed. Coloured Doppler Scan can be utilized for assessing the materno fetal blood flow through the placenta [2].

Fetal intrauterine transfusion (IUT) is considered to be the best choice for treating any anemia stemming from various blood disorders in the fetus. In case of Rh isoimmunised fetuses this intervention is accepted as the standard method by which intravenous blood transfusion can be given to an anemic fetus [3].

With the usage of ultrasound imaging in the first trimester there has been encouraging effects and influences in the improvement of fetal diagnosis and treatment. Two dimensional and three dimensional scans for embryonic improvement [4, 5], early embryonic circulation [6, 7], uterine receptivity, formation of yolk sac [8], embryo implantation [9] and detecting ultrasound biochemical disorders have become possible in the first trimester itself. Ultrasound imaging has immense application for invasive procedures like amniocentesis (which is best performed in the first trimester due to the abundance of amniotic fluid) chorionic biopsy, cardiocentesis and funiculocentesis [10].

Basic understanding of the fetal physiology and anatomy like renal function [10], fetal cardiac pathology, fetal hearing [11] and even behavioral changes of the fetus [12] by the help of ultrasound imaging techniques are also now possible. Certain invasive and cervical procedures include the transvaginal scan to detect preeclampsia of the fetus and karyotyping for fetal chromosomal problems. This process is also known as Chorionic villus sampling (CVS) and is usually done at 11–14 weeks of the first trimester period using placental tissues [13]. However the above invasive procedures are associated with a 1 % chance of miscarriage. Transvaginal USG can be also employed in the mother during the first trimester to assess fetal malformations occurring due to chromosomal aberrations. In the first trimester the sensitivity of this diagnostic tool is around 35 % to a maximum of 65 % or more and in the 20th week of the second trimester it is more than 80 % [14, 15].

Physiological midgut herniation, ascites, fetal edema or hydrops fetalis and some degree of hydrocephaly can be also detected in the first trimester. However not all anomalies can be detected in the first 3 months of development by imaging techniques as the fetus is still immature.

Fetal Down's syndrome, another major genetic disorder that is frequent among fetuses of mothers who possess a great risk of genetic aberrations can be detected in the first trimester by using three dimensional ultrasound imaging of the nuchal structures although there are many practical problems associated with this method. Total nuchal structure determination in the first trimester by three dimensional imaging was first introduced in 1998. In the first trimester there are often mistakes in identifying the exact anatomy which might be due to the limitations of the operator technique. Mistaking the amnion for the nuchal skin or measuring the nuchal translucency with fetal head flexed or extended can lead to wrong diagnosis of the disease. Certain set of parameters are required for diagnosing fetal Down's syndrome like appropriate image magnification, precise caliper placements and the optimal positioning of the fetus. However this diagnosis sometimes becomes extremely difficult in the first trimester as the anatomical status of the fetus still remains in a developmental phase. Also the age of the mother is a vital criteria for detecting Down's syndrome in the fetus [16]. Incidence of brain herniation mass due to encephalocoele in fetus is around 1–4 in 10,000 live births. Diagnosis of this disease in the first trimester might be impossible since the tumor size of the fetus will be small and can be often confused with some other anatomical anomalies thereby leading to a wrong diagnosis. Also at the end of the first trimester and the beginning of the second trimester meninges maybe the only structures protruding out to the exterior part of the brain thereby having the appearance of a cyst and can be easily confused with that of a perinatal brain tumor [17].

Megacystis, urethral obstruction and anomalies of the urethral valve, hypertrophic bladder,

dysplastic kidneys, ureteral dilations and absence of abdominal muscles can be easily detected by 3D USG in the first trimester [18]. Other neurological problems associated with failure in closure of the neural tube can be determined in the sixth week of the first trimester development. Also different pulmonary herniation and cysts can be diagnosed in the first trimester as the diaphragm completes development by the 9–12th week of fetal development. Defects of the fetus like vertebral ossification by guided USG scan can be determined in the second trimester as the vertebral development completes its formation between the 10th and 12th weeks of the first trimester [19].

Diagnosis of holoprosencephaly is also possible during the tenth week of the first trimester [20].

Anomalies of the umbilical cord like a single artery formation or hematomasis are, however, tough to detect in the first trimester as the full maturation of this cord happens during 12–14th week. This anomaly can be detected by Doppler angiography in the mid second trimester phase.

Fetal hands, fingers, feet and toes can be detected in the late first trimester with the help of 2D USG with a diagnostic efficiency of 12–41 % and around 41–65 % in case of a 3D USG system [21]. Facial malformations can be detected between week no.12 and 20 with 3D imaging along with the whole fetal face and body. Other anomalies like changes in the ductus venosus blood flow velocities can be also assessed in the first and early second trimesters [22–26].

MRI of the fetus can be used mainly to detect any fetal central nervous system abnormality along with any associated shortcomings as it is a safe, non-invasive process. MRI scan of the fetus can be extremely helpful in detecting cases of non conclusive complex CNS malformations also [27]. However applications of MRI in the first trimester are extremely limited except in cases where the assessment of the pregnant mother's health or voluntary interruption of pregnancy by the mother herself is essentially required. Use of MRI in the second trimester can be performed to detect any anomaly of the fetus along with ultrasound examination. Since the last two decades, the role of ultrasound screening in the first and second trimesters has not been emphasized by medical the community due to undue risks that might be associated with fetal growth and development. The American College of Obstetrics and Gynecology (ACOG) does not endorse its use in the initial stages of pregnancy due to ethical values and exploitations that might be associated with peri natal screening in many countries. However it is still widely practiced because ultrasound imaging is the most effective way by which early detection of fetal anomalies can be diagnosed. Also a study revealed that mothers who underwent a second trimester USG imaging favor the use of this tool also in the first trimester, as it will help them to opt for an early abortion in case if they want to opt for such processes [28].

Effect of Drugs, a Serious Contender for Making the Fetus a Patient in the 1st and 2nd Trimester

Both excessive drinking and heavy drug use have a massive effect on the fetus especially during the first and second trimesters. In the first trimester along with organogenesis, morphogenesis is also initiated. In the first 8 days before implantation, the embryo is known to be in a stage also termed as "all or none phase" where any damage during this phase will result in embryo abortion. However if the blastocyst can manage to escape and negate the harmful effects of toxins or drugs during this stage then it can undergo the process of implantation and further proceed for the growth and development without any anomalies associated. However it is always advised by physicians that abuse of drugs in a non-scientific manner can have a profound effect on the neurobehavior of the fetus, delay in synapse formation of the CNS and other teratogenic effects. ACE inhibitors used for treating hypertension in mothers were previously thought to be teratogenic during the second trimester, however now it has also shown to increase the chances of fetal malformations in the first trimester having a direct effect on the fetal CNS system [29].

The Australian health authorities have published a guideline detailing the effect of different drugs during the various stages of trimester development [30]. Cardiovascular drug Angiotensin II Receptor Antagonists (ARAS) has no reports

for fetal adverse effect during the first trimester which is probably because of lack of clinical data. However pregnant mothers taking such drugs should be changed to anti hypertensive drugs in order to maintain a normal blood pressure. Drugs like Phenindone D and Warfarin clot dissolving agents can lead to embryopathy of the fetus in the first trimester itself [30].

Lithium toxicity from Lithium salts D in the first and second trimesters can prove fatal to the fetus also. One to 2 % of neural tube defects like Spina Bifida have been seen to be associated with sodium valproate in the first trimester of pregnancy. Other allergic and immune system drugs like Levocarbastine B3 have shown to cause teratogenic results in animal models during the first trimester [30]. Nicotine as a chewing gum and cigarette smoking can cause harm to the fetus in the first trimester if it is exposed to its effect for long period [30].

Azole anti-fungal drugs can also lead to potential teratogenesis of the fetus due to aberrant sterol metabolism [53, 54]. Exposure to such drugs in the second trimester can often result in the fetus undergoing dysmorphic anomalies like hypoplasia of the nasal bones, craniosynostosis, bowed tibia and femur, cleft palate humeral radial fusion [31].

FDA has established five categories of drugs namely the A, B, C, D and X based on their level of toxicity and teratogenicity to the fetus. 'A' category shows no fetal risks, 'B' also shows no toxicities to humans but has shown adverse effects in animal studies whereas 'C' refers to adverse fetal effects in case of animal models but not in case of humans. 'D' shows fetal risks but can be prescribed in cases where it is necessary to outweigh the risks in order to impart beneficial effects. 'X' is where drugs can cause fetal toxicities and outweighs the patient benefits and induces unnecessary risks to the fetus and the mother [32].

Despite widespread concern about drug toxicity to the fetus it has been reported that only 2–3 % of all fetal congenital malformations and anomalies are directly related to the adverse effect of drugs. In fact genetic, environmental and other unknown causes have been reported to be some of the leading causes behind fetal anomalies. The passage of drugs through the placenta to the fetal compartment is actually dependant on a number of important factors like the molecular weight and size of the drug (less than 500 Da drugs can easily pass but not drugs of high molecular weight), its physico-chemical and biological properties. Not all drugs can pass through the placenta in the first and second trimester of fetal development as the placenta remains intact and allows only selective exchange of molecules across the placenta.

Also the fetal age is an extremely important criteria for drug toxicity or drug induced toxicity to the fetus. As discussed elsewhere in this chapter, drugs in the first trimester especially before the implantation of the blastocyst till the 20th day of fertilization which is also known as the "all or none stage", can either abort the development of the embryo or it can undergo successful implantation due to protective mechanisms in place without any adverse events. Teratogenesis is seen at a maximum between the third and eighth weeks of post fertilization. During the second trimester when organogenesis is nearing its end no observable level of acute toxicity of drugs to the fetus can be observed due to enhanced functions of the fetal liver and kidney, two main centers for drug metabolism and excretion in humans. However some potent drugs still might be able to alter the growth and development of the fetus in the second trimester although teratogenesis is unlikely. With increase in placental metabolism doses can be higher which might have a direct effect on the fetus also.

A study was conducted regarding the effects of anti retroviral therapies like acyclovir, valacyclovir or faciclovir during the first 3 months of 1,804 early pregnant mothers in the first trimester who received ARV therapies against a sample size of 830,000 [33]. It was found that the percentage of major birth defects in case of the 830,000 infants were 2.4 % or 19,000, and in the case of the 1,804 pregnant mothers who received anti retroviral therapies in their first trimester, it was found that 2.2 % or 40 infants had major birth defects [33]. Therefore the study confirmed that there was not a significant difference in the number of major birth defects between the ARV group and the normal group thereby concluding

that it is not always the effect of drugs that induces fetal toxicities and the usage of certain drugs in certain periods of gestational time with safe doses can be done without imparting any harm to the developing fetus [33].

Adverse events like teratomas are often associated with drugs taken in the first trimester, that is, when organogenesis and morphogenesis occur side by side [34], whereas in the case of second trimester adverse events of drugs are often associated with intra uterine growth reduction, bleeding and infections [35]. However some of the important criterias on which the fetal effect depends are the type of drugs used by the mother during pregnancy and at what stages, duration of exposure to the drugs and the gestation phase of the fetus [36].

Anti-tubercular Drug Mediated Toxicity to the Fetus in Early Pregnancy

Treatment of tuberculosis of the mother with Paraamino salicylic acid in the first trimester which is classified as a C class drug by the USFDA during pregnancy can result in congenital abnormalities to the fetus. Therefore it should be used in pregnant women only when there is no alternative treatment option available with any other suitable drug that can affect the fetus to a minimal level [37, 38]. Other C category drugs like fluroquinoles can induce fetal toxicities like damage to the articular cartilage, arthritis, lesions in animal models, [37, 38] ethionamide which can cross the placental barrier and has shown to induce fetal malformations [39, 40]. Aminoglycosides on the other hand are D category drugs which can rapidly cross the placental barrier and induce ototoxicity and nephrotoxicity [37, 38, 41].

Teratogenic Effect of Drugs on the Fetus during 1st and 2nd Trimesters

Evidences for the teratogenic effects of most anti neoplastic drugs in the first trimester are little as very few women are detected with cancer in this stage. However with limited data available, it has been noted that chemotherapy during the first trimester is often associated with teratogenic effects on the fetus and causing malformations. The most vulnerable and susceptible period of teratogenic effects of cancer drugs in the first trimester is generally between 2 and 8 weeks with a high risk of 20–30 % fetal teratogenesis [42].

Chemotherapy has a detrimental effect on the development of the fetus during the first and second trimester. During the first trimester, i.e., at about 17 days of post conception in the preembryonic period rapid division of the cells are observed and any chemotherapeutic toxicity during this stage can lead to abortion. On the other hand there are many protective and preventive mechanisms in place, which may help the developing embryo during the 2–8 weeks phase to escape the chemotherapeutic drug toxicities and may lead to organogenesis and the successful development of the fetus without anomalies [43]. However any end organ damage to the developing and immature heart, lung, renal system, limbs and neural tube can be irreversible in nature during the first and second trimester period.

During this period of development the renal and gastrointestinal tracts of the fetus along with the cerebral cortex remain highly susceptible to chemotherapy induced drug toxicity which can often prove fatal for the fetus [43]. It has been noted that there is an increased risk of around 10–20 % of fetal abortion, inhibition of blastocyst implantation and trophoblast migration in the first trimester if the chemotherapeutic drugs are exposed to the fetus [44, 45].

Continuous exposure of the fetus to cancer drugs in the second trimester can further complicate things leading to Intra uterine growth retardation (IUGR), fetal pre term delivery but no increase in the case of congenital anomalies [46]. However genetic predisposition factors also play an important role in fetal toxicity in respect to cancer drugs as it has been observed that all chemotherapeutic drugs do not express their adverse effects and toxicity levels on the developing embryo at the same level as they do in some other embryos [47].

In case of slow going cancers it is better to advice the patient to wait till the second trimester to start chemotherapy during which a multi drug treatment can be started. However in cases where the chemotherapy should be initiated from the first trimester it is advisable to start with a single dose of safer chemotherapeutic drug like a vinca alkaloid or anthracycline. Unfortunately in a real life scenario in order to assess the risks of the mother's cancer it is often not an easy choice to delay the chemotherapy regimen till the second trimester as it might bring in more harm to the mother's as well as the fetus' health [42]. However it has been noted that most babies where the maternal chemotherapy was started during the second trimester and beyond were born healthy without any structural malformations [42].

One of the most common non steroid anti-inflammatory drug (NSAID) used by pregnant mothers is Indomethacin [48]. It is an analgesic used commonly to treat symptoms of rheumatoid arthritis, ankylosing spondylitis and osteoarthritis. Indomethacin functions by inhibiting the activities of cyclo-oxygenase enzyme. In animal models it has been observed that Indomethacin can cross the placenta freely during the second trimester onwards whereas in the first trimester the passage is minimal [49–51].

Regarding its use in humans, Aselton et al. (1985) reported that 1 congenital defect was observed in 50 women who had taken this drug during their pregnancy [52]. Also another report revealed that nine babies of eight mothers who had consumed Indomethacin in the first trimester had a mild degree of hypospadia and no adverse reports were reported in the remaining group [53]. In a large study conducted at Michigan with a sample size of 229,101 pregnancies over 5 years where the mother had consumed Indomethacin prescribed during the first trimester, only 6.1 % of birth defects were observed [54].

Studies by Moise et al. [50] revealed the noticeable passage of Indomethacin in the second half of pregnancy from the second trimester onwards. Indomethacin has profound negative effects on the fetus like constriction of the ductus arteriosus leading to tricuspid regurgitation in utero and resulting in hydrops fetalis and fetal death [55, 56], decreased urine output [57], and oligohydraminos [58].

Radiotherapy Induced Fetal Anomalies

Incidence of cervical cancer, Hodgkin's lymphoma, breast cancer and melanoma are quite frequent at the time of pregnancy. Radiotherapy often used to treat such cases of malignancies can result in fetal abortion if the abdomens of pregnant women are not properly shielded. Abdominal shielding can reduce the risks of fetal death by 50 %. However, in most cases, radiotherapy in the pregnant mother is contraindicated. Chromosomal abnormalities can be one of the leading causes behind miscarriages and fetal abortions in the first trimester. Progesterone deficiencies also result in a high incidence of fetal deaths in the second trimester. Also 15 % of pregnancy losses in the second trimester can be attributed to other factors like uterus malformations, uterine fibroid formations and other cervical problems which might also contribute to premature birth [59].

Are Anti Retroviral Drugs Safe for Use in the 1st and 2nd Trimesters?

Use of antiretroviral drugs during the first trimester of pregnancy is extremely harmful for the fetus. This is the time of organogenesis when the ectoderm, endoderm and mesoderm form internal organs. Drug toxicity during this time results in the most severe birth defects. The Antiretroviral Pregnancy Registry, an international database compiling data on HIV+ pregnant women and antiretroviral treatment, reports that the rate of birth defects in children exposed to ARVs at any time in utero is 2.7 % [18]. Children exposed to ARVs during the first trimester show a slightly higher incidence of birth defects at 2.9 % [60].

Therefore the need to follow certain standard guidelines for application of ART in the first

trimester is always recommended. Based on ethical considerations a clinician has to assess the risk benefit ratio of the mother and the fetus and is encouraged to start ART's from the first trimester in cases of severely infected mothers who have a high risk of transmitting the disease to the fetus [61].

Risk of late acidosis [62, 63] with certain drugs like Didanosine (ddl) in second line RT regimens should be avoided in pregnancy as it can lead to fetal and maternal deaths. A mother may be given didanosine (ddl) in second-line ART regimens. Exposure to AZC and 3TC drugs in the first trimester has shown around twofold increase of risk to the fetus. 6.3 % birth defects were observed in live born infants who were exposed to ddl drug compared to just 1.1 % in cases of late exposure to this drug [64]. Also a French study reported that the combinatorial treatment of AZC and 3TC given to women during the second trimester period of fetal development resulted in a high rate of fetal anemia, neutropenia when compared to infants who were only exposed to AZT [65]. In cases of oral administration of calcium channel antagonists like verapamil, nifedipine in pregnant mothers during the first trimester for treating hypertension did not show any teratogenic risk to the fetus [66].

Fluconazole should be avoided during the first trimester of pregnancy as there are four reports of craniofocal and skeletal congenital malformations at high doses during the first 3 months of pregnancy [67, 68]. However in low doses no malformations have been observed in more than 800 cases of pregnancies [69–72]. Rifampacin a very potent anti tuberculosis drug should be also used with caution especially in the first trimester as it may have teratogenic effect on the fetus. Use of Clarithromycin in animal models has been associated with major malformations but in human studies comprising a study group of 265 women showed no evidence of teratogenicity [73, 74]. Also, in Toronto, Canada, 123 patients when treated with azithromycin in the first trimester, no increase in fetal malformations were observed when compared to a non-teratogenic antibiotic drug [74]. Also there is a huge concern regarding the safety of trimethoprim sulphamethoxazole which is the preferred prophylactic drug for Pneumocystis jirovecii or PCP regarding its use in the first trimester [75–77].

Physiological and Anatomical Disorders of the Fetus in the Early Stages of Pregnancy

Malformations of the brain are usually associated with the first and second trimester of pregnancy. Lesions in the white matter of the fetus has been observed between the mid second trimester to the middle of the third trimester as this is the time when the functional and structural maturation of the brain takes place [78, 79].

Insulin like growth factors-1 and 2 are extremely important in the initial phases of growth and development of the fetus as they are directly related to the fetal size and brain development [80, 81]. Excess secretion or rather passage of insulin can result in conditions like macrosmia [82] whereas underproduction has found to be associated with congenital agenesis of the pancreas [83]. Diabetes mellitus in neonates has shown to induce growth retardation in both animal and human systems due to depletion of the beta pancreatic cells further leading to cell cycle arrest and reduction in angiogenesis thus limiting the overall capacity of the fetus to produce insulin [84]. It should be noted that maternal IGF-1 and 2 do not cross the placenta directly. They instead have shown to interact with the placenta and help in establishing a feto placental unit and by the help of the IGF binding proteins and proteases, insulin like growth factors are delivered into the placenta [85]. Thyroid and sex hormones have little effect on fetal growth and development. Hormone leptin has shown to help in the growth of the fetus in the initial phases of pregnancy. Also in the absence of growth hormones, it has been seen that the fetus tends to be small in size [86].

Intrauterine growth development another common complication observed during pregnancy is often found to be associated with the

second trimester. Any deformity or harm to the placenta can result in its integrity getting destroyed and reduction in its size leading to an extreme adverse environment for the fetus. Preclampsia, a very common condition in the mother, can result in the decrease of placental growth, leading to development of less number of terminal villi. This further results in to a severe reduction in the metabolic rate by cutting down the level of oxygen and glucose transport to the fetus. Due to this there will be a direct effect on the metabolic activity of the fetus in the organogenesis period as there will be already a huge demand for high energy to perform various physiological activities and functions. This failure of the placental layer will have a severe effect on the development of the fetus in the first and second trimesters [87]. Other factors like multiple gestation, chromosomal anomaly, fetal infection or toxicity and delivery of toxic and harmful substrates can also induce intrauterine growth reduction (IUGR) among neonates in the first 6 months of pregnancy [88].

IUGR has direct consequences on infants who are born with a high hematocrit. This is an erythropoietic response to hypoxic events due to insufficiency of the placenta. Hypertension in mothers is also related to thrombocytopenia and neutropenia in the fetus [89]. To prevent IUGR or to detect the fetal viability and its gross malformations an ultrasound examination is performed normally during the first 8–10 weeks of gestational period [90]. Physical measurements like symphysis-fundus height (SFH) are also recommended when USG is not available. A study in The Netherlands revealed that starvation of the mother during the first phases of pregnancy can also lead to IUGR [91].

Fetuses of Insulin dependent diabetic mothers normally have two to eightfold risks of teratogenesis [92, 93] in the CNS like Spina bifida, heart, kidney and skeleton like caudal regression [92–94]. Grave's disease, Systemic Lupus Erythromatosus, rhesus allo-immunization, anemia due to hemolytic disease of the newborn are also commonly associated with second trimester pregnancy due to transplacental passage of maternal antibodies. Fetal hypothyroidism is linked to growth retardation and advanced bone ageing and craniosynostosis [95].

Ischemic lesion of the cerebrum can cause schizencephaly of the fetus which is accompanied by the presence of abnormal grey matter particularly in the gyra [96]. Injuries due to trauma or accidents during the 24th week or end of second trimester can lead to cystic lesions of the brain with smooth walls devoid of gray matter line and clefts in the brain [96].

Introduction to Hematological Disorders of the Fetus and in Utero Transfusion

Forty years ago the fetus would have been conceived as a mere passenger in a closed and restricted environment inside the mother's womb. With the advent of two and three dimensional and real time USG scans it has been possible to consider the fetus now as a patient in terms of medical definition although ethically it is still debatable. In the late 1970s the fetus's hematologic stature could be assessed only through the bilirubin level biomarkers obtained from the amniotic fluid. In 1973 Valenti used a 27 gauge needle and punctured the chorionic blood vessels under direct visualization [97]. This procedure came to be known as fetoscopy [98] which is now restricted in its use due to a 5 % fetal mortality rate. In 1983 Daffos and his group further reported a new minimally invasive diagnostic technique with the help of real time USG guided imaging whereby a 20 gauge needle was directly inserted into the umbilical vessels of the fetus; a low mortality rate of 1 % was reported [99]. Also as time went by different parameters for assessing fetal blood disorders also changed, thereby giving rise to a new field of medicine also known as fetal hematology where the fetal hematocrit and the leukocyte count became some of the important parameters to assess the well being of the fetus. The fetal hematocrit and the leukocytic count increases with gestational time whereas

erythroblasts and reticulocytes count decreases [100–102]. However the fetal platelet count remains more or less stable during the whole pregnancy period without much observable changes [100].

Liley in 1963 was the pioneer of the first successful treatment of hemolytic disease of the fetus when he introduced the concept of intraperitoneal transfusion [103]. In 1981 Rodeck et al. modified the technique a bit and the first intravascular fetal blood transfusion (IUT) using a needle into the chorionic plate vessels directly under visualization or fetoscopy was performed [104].

Another group from Denmark, the following year, reported an intravascular transfusion of a fetus by puncturing the umbilical cord under the guidance of USG [105]. Since then many researchers have shared their different thoughts and opinions in the process of intra uterine blood transfusion and serious efforts are still constantly being undertaken in order to upgrade the current standard of treatment. Over the years with the evolution of fetal therapies, the intraperitoneal transfusion technique was abandoned. The intravascular technique became the standardized intervention process and therefore it became a major technical advancement in the field of fetal hematology and surgery. Survival of hydropic fetuses was increased by the intravascular mode of blood transfusion although it is still not clear whether this process would be an efficient one in case of non hydropic fetuses [106]. Through this IUT it has been possible to treat different disorders of the red blood cells such as red cell alloimmunization, fetomaternal haemorrhage and parovirus infections. Fetal thrombocytopenia occurring due to platelet alloimmunization in the fetus has also been undertaken through this IUT process.

Due to the presence of the blood placental barrier, there is no mixing of the maternal and fetal blood but different FACS analysis has shown the presence of small fetomaternal hemorrhages in most cases of pregnancies. When the fetal erythrocytes with their fetal red cell antigen enter the maternal circulation, formation of antibodies takes place mediated through a process also known as red cell alloimmunization. The placenta plays an active role in transferring the IgG antibodies via itself into the fetal circulation thereby causing antigen and antibody reactions in fetal erythrocytes resulting in cell medicated destruction of red blood cells especially in the spleen, which decreases the rate of RBC production and ultimately causes fetal anemia. Apart from Rh and D antigens, there are 43 other antigens that are implicated in hemolytic disease of the new born like Duffy, Kidd and Kell. However the intravascular transfusion of erythrocytes too has certain shortcomings like the formation of cord hematoma, fetal bradycardia, wide variable swings in the parameters between transfusions and formation of porencephalic cysts. A combined technique of intraperitoneal and intravascular transfusion also revealed this to be a much better process than either of the two when applied individually. It resulted in a more stable hematocrit and allowed longer intervals between each transfusion to the fetus. The objective of one study was to reach a hematocrit value of 0.35–0.40. The authors did an intraperitoneal transfusion in fetuses of gestational age 20 weeks along with a dose of vecuronium (0.1 mg per kg of USG estimated fetal body weight) before the start of the next intravascular transfusion in order to stabilize the fetus movement for 2 h thereby reducing the chance of fetal injury during the process. Every 2 weeks interval, transfusion was undertaken for the first two sittings and then the interval period was further increased to 3–4 weeks time. Hydropic or severely anemic fetuses of the early second trimester do not tolerate intravascular transfusions compared to fetuses of older gestational time. The mean end point of the hematocrit which is often regarded as an important biomarker for hemolytic anemias in case of early second trimester fetuses is normally between 0.20 and 0.25 in case of first transfusion and 0.35 in cases of second transfusions to achieve a normal level of hematocrit function [107, 108]. Some clinicians look for a fetal hematocrit value of 0.50–0.65 via intravascular transfusions whereas in case of the combined

intraperitoneal and intravascular transfusions, a fetal hematocrit value of 0.35–0.40 is often regarded as a standard.

In 1 out of 1,000 births, fetal mortality occurs due to substantial fetomaternal hemorrhage and around 1 in 800 results in fetal morbidity. In a study of five intrauterine transfusions, three such cases reported complaints regarding decreased fetal movement due to fetal bradycardia, sinusoidal heart pattern, falling hematocrit levels due to continuous fetomaternal haemorrhage after IUT and in one case hydrops fetalis was observed via USG. However pregnancy was prolonged in one of these five cases where three received a single intrauterine transfusion and two received double intrauterine transfusion. This one case where pregnancy prolonging was reported after an intraperitoneal transfusion during the 21st week helped in reversing the condition of hydrops fetalis with a normal baby delivery at 34 weeks of gestation. However due to the low success rate where only one fetus responded to the IUT as observed in the case of five fetuses the safety and efficacy of this intervention is still questioned.

Ten percent of in utero intracranial hemorrhage has been documented as early as second trimester [109, 110]. The clinician becomes aware of this hemorrhage or fetal thrombocytopenia only after the infant is born thereby resulting in a high mortality rate at the time of birth [111]. Regarding initial evaluation of the disease it has been suggested that cordiocentesis as early as 20 weeks during the second trimester can be conducted in order to assess the degree of fetal platelet count and antigen status. However this approach of cordiocentesis is also associated with high mortality as 14 cases of fetal death caused due to fetal hemorrhage during blood sampling of this disorder have been reported [99]. Maternal platelets if transfused during this time can help in coagulation of the blood thereby stopping the loss of excess blood from the site of puncture or else previous analysis of the fetal platelet count should be undertaken before embarking on this method.

Infection of the Fetus during the First and Second Trimesters

Infections of the fetus can be vertically transmitted from the mother to the fetus which includes mycobacterium tuberculosis [112, 113], cryptococcal infections [114, 115], Pneumocystis jirovecii or PCP [116, 117], Cytomegalovirus (CMV) and toxoplasmosis gondii [118]. Other factors like the intactness of the blood placental barrier, the time of gestational period and the type of pathogen that is infecting the mother and its potency also plays an important role in transmitting the infections from the mother to the fetus thus making the fetus a patient.

Toxoplasmosis gondii is mainly acquired from uncooked raw meat and cat feces. The risk of fetus infection in the first trimester is 15 % when compared to 45 % in the second trimester. However it is seen that in mothers who get infected in the first trimester, there is 80 % transmission of the infections to the fetus in the later stages, whereas it is only 50 % if acquired by the mother during the second trimester. However infections in the mother in the later stage can cause fetal damage or still birth with damage to the CNS in a live infant. Spiramycin treatment of the mother has shown to reduce the incidence of vertical transmission by 60 % [119]. The above phenomenon might be due to the fact that in the initial stages of development the placental barrier is extremely intact thereby not allowing many pathogens to cross the blood placental barrier and enter the fetal compartment. This mechanism of placenta is in place to support the immature fetal immune system. However if the maternal infection is not treated in the initial stages then there are high chances that the pathogen might be able to cross the placental barrier when it loses its intactness due to cellular devascularization in the later trimester phases. Although the fetal immune system also gradually develops by the time it reaches its 14th week still it remains too immature to combat the virulency and pathogenicity of many infections.

Cytomegalovirus (CMV) is another common cause of in utero infections and thereby screening for this virus is extremely essential. Around 50 % of women of child bearing age remain susceptible to this virus and only 1 % can have the risk of getting affected by this virus. These 1 % women getting affected with CMV can transmit 45 % of this virus to the fetus in the initial stages of pregnancy thereby posing an immense risk to the fetus. Infections like Herpes Simplex Virus (HSV) are extremely rare in terms of in utero infections but if it is transmitted to the fetus it can cause severe damage to it at any stage. Genital herpes which usually causes cold sores has little risk of transmission to the fetus if it infects the mother in the first and second trimester [119].

Human Parovirus B 19 can affect around 2.5–38 % of fetuses and inhibits fetal erythropoiesis resulting in the development of aplastic anemia, non immune hydrops and even fetal abortions [120, 121]. Maternal infection is assessed by the presence of a specific IgM antibody which appears 3–4 days after the onset of clinical symptoms and continues to persist for another 3–4 months [122]. Within this window period of 4–6 weeks time after the maternal infection, the fetus might acquire this infection which can be detected by means of USG imaging weekly. Diagnosis of the Human Parovirus infection in the fetus can be assessed by the presence of fetalis hydrops followed by severe anemia in some cases, a negative direct Coombs test, an inappropriately low reticulocyte count, elevated levels of total IgM and hepatic enzyme levels [123], presence of viral particles in the fetal ascetic fluid [124] or the presence of viral DNA collected from fetal fluids. Such fetuses undergo a serial intrauterine transfusion process at short intervals ranging between 1 and 7 days. Increase in the fetal reticulocyte count signals a recovery of the fetal bone marrow and further reduces the chances for intra vascular uterine transfusions. Enhanced intrauterine transfusions among anemic Rh positive fetuses can result in the suppression of the fetal erythropoiesis and lead to the dependence of the fetus on serial procedures in order to maintain the hematocrit level. Non immune parovirus infections may require weeks of transfusions to correct the fetal hematocrit level to a normal one. Hepatits B virus can be acquired in utero by the fetus through maternal infection or during the course of fetal development. Klein et al. demonstrated evidence that there is a causal relationship of rubella, cytomegalovirus and toxoplasmosis infection with IUGR [125].

Primary Varicella Zoster in the first and second trimester of pregnancy may result in the birth of a fetus born with congenital malformations. This is also referred to as the 'Congenital Varicella Syndrome' which is rare affecting only 2.8 % of women with chicken pox in the first 20 weeks post conception. The maximum risk of getting infected with this pathogen is however between the 12 and 20 weeks of gestational time. The fetus might develop symptoms like limb hypoplasia, ocular abnormalities, mental retardation and pre mature death. Varicella Pneumonia another sub group of this infection can be highly infectious during the third trimester of fetal development. Normally systemic antiviral medication is prescribed [126].

In case of HIV, viral transmission from mother to fetus is around 25 %. The fetus is more likely to acquire the disease if the mother has a high viremia or a low CD34 positive cell count. The risk of congenital infection in case of Rubella is also very high to the fetus in case of the first trimester. However between the 23rd and 26th gestation week the risk of the transmission of this disease decreases to around 25 %. The first 11 weeks of the trimester can produce teratogenic effects to the fetus; however, no such risks are associated with the fetus once it crosses the 16 weeks of the second trimester phase [127].

Bacterial infections like Group B streptococcal (GBS) can be one of the main life threatening infections among the new born as it has the potency to induce pneumonia infection; if acquired by the mother in the first 2 weeks of pregnancy in the first trimester it can result in the

fetal hearing and vision loss too. GBS is also found in 20 % pregnant women in their vaginas during the last trimester and this has the potentiality to affect the child during birth. Most of the bacterial infections however infect the fetus in the later stages of pregnancy or post natal [127].

Experimental Treatment Options for the Fetus in Utero during the First and Second Trimester of Growth and Development

Stem cell therapy in utero of the fetus is an extremely new and a very exciting concept in treating different disorders of the fetus. It holds great promise in treating many fetal complications. The reason why stem cell transplantation in the late first and second trimester and beyond can be a very exciting opportunity is because the immunological system of the fetus is still immatured during this stage and it fails to recognize foreign antigen or cells thereby reducing the chances of abortion and other immune mediated rejections. The size of the fetus is another important criteria as on it is dependent the dosage regime [128].

In sickle cell anemia stem cell therapy is an extremely good option. Aetiology of sickle cell anemia is because of the change in the shape of the erythrocytes which become crescent shaped and a decrease in their life span compared to normal blood cells. They also tend to clot small blood vessels [128]. Normally the onset of anemia is seen in the fetus during the first 4–8 weeks of gestation in the first trimester [129].

Scientists like Flake are currently investigating the potentials of blood forming stem cells to treat sickle cell anemia as the delivery of stem cells through intrauterine transfer involves minimally invasive surgery and is thereby less risky [128]. These blood stem cells can be co-cultured in the lab under in vitro conditions by taking half from the mother and half from the father. The fetus's weakened immune system especially in the first and second trimester might take up the cells thereby making it a chimeric and a tolerant fetus to future donors thus increasing the chances of accepting more stem cells from the same donors in future especially during post natal development.

Use of Umbilical Cord Blood as a Viable Substitute and Option for Intrauterine Transfusions

Umbilical cord blood has shown immense promise in treating thalassemia, anemia and other blood related disorders and malignancies. Bhattacharya et al. have shown encouraging reports of cord blood transfusion in more than 1,000 patients suffering from different ailments [130]. New concepts in this area stem from this very thought of using whole umbilical cord blood transfusion in treating fetal anemias and blood disorders. Volume of the cord blood transfused, it is conceptualized, is an important criteria for treating the fetus. Normally for neonates of extremely low birth weight, approximately 10–20 ml/kg of body weight, one or two bottles of autologus blood transfusions can be sufficient, but for the fetuses in the first and second trimester, dose is a big factor to make sure that excess of dose will not result in abortion as the fetus in the first two trimesters, is extremely prone to abortion in the presence of foreign molecules and cells [131, 132]. Umbilical cord blood transfusion is also an alternate but feasible option for treating premature deliveries where the fetuses are of the age of 27 or even 24 weeks of gestational phase. Several clinical trials have also shown the importance of umbilical cord blood transfusions obtained from both autologus and allogeneic sources [133, 134].

The procedure in doing so will involve the aid of USG guided image for proper transfusion of the fetus in utero. The inherent properties of cord blood like a higher fetal hemoglobin which is almost 50 % more than that of adult hemoglobin and a larger capacity to transport and release oxygen after transfusion due to higher oxygen dissociation curve makes cord blood an attractive option for intrauterine transfusion [135, 136]. Platelet content in cord blood is not so high. Also presence of small amounts of pro and

anti-coagulant factors in the plasma of the fetal cord blood makes it an important alternative to adult blood transfusion as it helps in reducing the chances of coagulation post intra uterine fetal transfusion. This is also one of the reasons why neonate plasmas are not efficient in correcting bleeding due to the absence of coagulating factors. In case of allogeneic cord blood transfusion in adults normally no HLA matching is required apart from the standard blood group matching. It has been found in most patients that after transfusion there has been a rise of CD34 positive cells in the peripheral blood. If this is true in case of the adult immune system where it is highly developed and specialized to recognize between self and non self antigens then this same principle would be true in case of the fetus also where the immune system is naive. Umbilical cord blood can be stored for 35 days; however after 2–3 weeks of storage the physiological potassium level of the cord blood increases leading to hyperkalemia and if transfused after 3 weeks time it can cause fetal cardiac arythmias [137].

Important disadvantage of cord blood is that the volume of cord blood collected is low and therefore it requires multiple units for a minimal effective level of transfusion dose. Chances of viral transmissions are also high especially during the term time but it has been shown through various studies that the risk of HIV transmission is between 1 in 100,000 to a million [138]. Risk of transmission of Hepatitis B is 1 in 50,000. This can be attributed to the fact that Nature's most dynamic screening system, the placenta, can selectively screen at the nano-molecular level thereby allowing the selective transmission of molecules and inhibiting the passage of pathogens through its fetomaternal barrier. However, there are still chances of viruses and pathogens to move through the placental barrier especially during the near term time as the fetomaternal barrier can gradually undergo cellular de-vascularization. This risk in adult blood is not perceived in the west due to stringent screening protocols that are in place but in developing countries like India, Bangladesh, Sri Lanka, Pakistan although there are standard operating procedures in place for proper screening of adult blood many of them are almost nonexistent. Also in the west molecular screening of the adult blood is conducted by means of PCR which is unlikely in case of developing countries due to high cost. Therefore cord blood plays an important role here as it is dynamically screened by the placenta itself. Another important factor in obtaining adult blood for transfusion purposes during emergencies especially in developing countries is that it is an extremely arduous and time consuming process. However in case of cord blood after a proper informed consent from the mother it can be easily available. Also cord blood is less thrombogenic. The erythrocytes of newborns do not express Kelly like antigens and other antigens like A and B, therefore cord blood can be less immunogenic causing no adverse event in the fetus after transfusion [129].

In case of stem cell transplantations it has been seen in mouse and lamb models that the fetus during its early stages of development has an excellent potentiality of accepting donor or foreign cells and has a unique tolerance to foreign antigens and also the capacity to transport large cell doses within a short time due to the small fetus size and also an efficient engraftment potential of the stem cells in the fetus [139, 140]. Successful haemotopietic stem cell transplantations have been observed and achieved in immunodeficient fetuses thereby suggesting the role of an immunological barrier that often exists in the early stages of pregnancy in the human fetal compartment [141]. However it was observed that allogenic responses from the 9th to 12th week started, which increased with gestational time ultimately leading to the theory of GvHD suggesting that although it might be considered that the fetus is immune resistant it can still elicit responses specific to stem cells, the mechanisms of which are still known [142]. In these cases the role of mesenchymal stem cells can be important as these groups of stem cells have excellent immune modulatory functions. Mesenchymal stem cells contain an potentials for treating adult osteogenic diseases, skeletal disease, cardiac diseases, pancreatic disorders leading to diabetes mellitus, liver regeneration, and even in some cases it has shown some potentialities to treat

neurological disorders but with inconsistent reports. However it is believed that in case of blood diseases even allogenic haemotopoietic stem cells (HSC) will be more potent and viable than mesenchymal stem cells (MSC) because of the HSC ontogeny and presence of many blood forming precursor cells [141].

Intrauterine stem cell transplantation has been performed in cases of haemoglobinopathies, osteogenesis imperfecta, immunodeficiencies but more clinical data is needed to substantiate the procedure's curative value. Tiblad and Westgren it was found that in the case of intervention of stem cells, source and type of the donor stem cells and the specific disease in question varies greatly between the first and second trimesters [143].

The same can be said in case of gene therapies where the introduction of a foreign viral vector can give rise to a cascade of immune reactions, but there can be an exception in the fetus during the first and second trimester as the fetal immunity is still in its nascent stage of development. Gene therapy can be applied by intra uterine delivery of the viral vector in the fetus to treat various genetic disorders like hemophilia A and B. It has already been shown that the previously deficient blood clotting factors in case of hemophilia tends to function normally after the introduction of the correct gene. Fetal lamb model has shown low mortality rate of 3–15 % after a minimally invasive USG imaging was conducted targeting the muscles, diaphragm, cerebral ventricles, peritoneum and liver via the intra uterine system. Therefore gene therapy can give a unique solution to many genetic diseases like congenital blindness, fetal cystic fibrosis prevailing in the early fetus. This mode of treatment can also help in stopping the irreversible damage to organs before or shortly after delivery among neonates [141].

Surgical Interventions of the Fetus during 1st and 2nd Trimester

Interventions in the fetus are extremely risky procedures as it can cause harm to both the mother and the fetus. A simple process like amniocentesis itself is associated with 0.5–1.4 % fetal mortality [144]. Open fetal surgical procedures often pose a greater risk of uterine dehiscence and rupture having a direct negative implication on the current and future pregnancy prospective.

Any rectification of the fetus in terms of anatomy and physiology can be conducted by means of two types of interventions: open fetal surgery and intra uterine therapy. Open fetal surgery is normally preferred during the second trimester whereas intra uterine therapies which include blood transfusion and stem cell therapies can be conducted between the late first trimester and early second trimester. Open fetal surgery itself is also a very risky procedure [145]. In 1990 under the guidance of Dr. Michael Harrison and his team at UCSF the first successful intervention for congestive diaphragmatic hernia of the fetus was performed [146]. This was soon followed by a prospective trial comparing open fetal surgical procedures to post natal repair in a sample size of seven patients in the case of an isolated left sided chronic diaphragmatic hernia with a significant displacement in the ipsilateral lung and intrathoracic stomach region. However the trial failed to show any prospective difference in survival between the two groups. Also the intervention in the fetal group had a much longer duration of hospitalization compared to the second group where this surgical intervention was conducted during the post natal stage of development [147]. Therefore clinicians target another alternative repair form known as the fetal tracheal occlusion intervention (FeTO) since open fetal repair has been rejected by many surgeons in favor of tracheal occlusion. However FeTO also had a mixed success as it has lost its momentum among North American clinicians following the UCSF trial. However in Europe the trend is quite different as more and more tracheal occlusions of the fetus are being treated by the FeTO way which has shown high survival rates among CDH fetuses reported to be at less than 30 % for left sided CDH and less than 45 % for right sided CDH [148, 149].

It is believed that tumors that gets manifested during the first 3 months of development normally get termed as congenital tumors which progress with increase in gestation time [150,

151]. The number of fetal neuroblastic nodules increase during the 17–20th week of gestation and are indistinguishable from the neuroblastoma in situ [152, 153]. Therefore the presence of neuroblastoma of the fetus in autopsy series is significantly higher than the actual clinical manifestation of the disease and the precise way to actually identify this condition is by the help of USG guided imaging. Surgery in such cases remains the standard operating procedure with a conservative detection and diagnosis of the neuroblastomas [154].

Hemangioblastomas, another class of solid tumors can be detected in a 16 weeks fetus and might have different sonographic appearances and can be either hypoechoic or hyperechoic or maybe even of mixed echogenicity. Fetal MRI or a color Doppler evaluation can be performed in such cases in the second trimester; these will show the increase of flow along with a flow void in the mass. Also in some cases fetuses may develop consumptive coagulopathy known as the Kasabach-Merritt sequence which if not detected in the early trimester can result in congestive heart failure of the fetus [155].

Sacrococcygeal tumors (SCT) are a rare form of neoplasms which are being diagnosed by USG in utero frequently and are often related to intra uterine fetal demise (IUFD). SCT's if not treated from the first to second trimester can result in abnormal size of the fetus resulting in cardiac failure and non-immune hydrops with vascular shunting. Tumors can rarely cause internal and external hemorrhages [156].

Spina bifida or myelomeningocele that occurs in almost 5–10 pregnancies in 10,000 women in the United States is a non-lethal neural tube defect. It occurs mostly during the third or fourth week of the first trimester however with improved peri natal screening the incidence rate has been reduced to 3.4 per 10,000 live births currently [157, 158].

Spina bifida involves the failure of the development of the neural plate and thereby fails to close along its length often leading to an open spinal canal condition with exposure of meninges and other natural elements of the brain. The aetiology of spina bifida can be due to the teratogenic effects of drugs like carbamazepine, pregestational diabetes or valproic acid exposure. Usually maternal serum alpha feto protein measurement and USG screening is conducted during the second trimester to assess the condition [159].

In order to treat spina bifida in the fetus surgical resection is required which can be performed through maternal hysterotomy and laparotomy. Normally open repair is required in cases of large lesions. However, risks are involved to both the mother and the fetus. Open fetal surgery in most of the cases results in pre term labour [160, 161].

Common cardiac lesions like intrapericardial teratomas and rhabdomyomas are not easy to detect in the first trimester mainly because of the immature anatomy of the fetus. Other cardiac lesions include teratomas, fibromas, myxoma and vascular malformations [162, 163]. The incidence of cardiac neoplasms are extremely less with an approximately 0.1 % of the fetus getting affected [164, 165]. Cardiac lesions of the fetus are usually detectable after the first 22 weeks of gestation by means of prenatal USG imaging techniques which involves the imaging of the pericardium, myocardium, cardiac valves and/or the major pericardial blood vessels [166–168]. The survival rates of cardiac neoplasms are quite low with 57 % chances of IUFD. The most common type of cardiac neoplasm observed in the fetal heart is the rhabdomyoma [166].

There can be a chance of sound and correct diagnosis of such cardiac lesions during the second trimester as that is the time when the heart of the fetus matures [169]. Also nuchal translucency, a diagnosis meant for Down's syndrome, can be prescribed in case of fetal heart diseases even if the fetus has normal chromosomes [170, 171].

In case of CHD, early first trimester fetal echocardiography conducted trans vaginally or trans abdominally cannot have the same degree of detection efficiency as in the case of second trimester for CHD as many forms of fetal CHD develop only during the late first trimester or during the course of the second trimester [172–174]. However it is always logical to start monitoring the fetus for any anomaly in the first trimester itself.

Some surgeons have opined that due to the presence of non-immune hydrops open fetal cardiac surgical resection through maternal hysterotomy and fetal median hysterotomy can be conducted. However till date there have been no reported cases of open fetal resection of a cardiac neoplasm.

Another congenital anomaly of the fetal cardiac system is Valvar aortic stenosis which represents the development of a thick valve during the second trimester. Severe cases of valvar aortic stenosis if present during the mid second trimester can potentially affect the peri natal development of the fetus's left ventricle resulting in a form of dilated cardiomyopathy [175, 176].

Other conditions like obstruction of the fetus and trachea can result in cardiac failure of the fetus along with craniofacial defects and cranial nerve injuries. Highly vascularized lesions can lead to fetal cardiac arrest too. Majority of the fetal lesions are comprised of cervical teratoma, cystic hygroma and other vascular malformations [177]. Rare defects of the neck like thymic cysts or congenital neuroblastomas can be also observed in the fetus during its initial stages of development.

Incomplete closure of neura tube defects can give rise to myelomeningocele resulting in an open spinal canal with exposed neural tissues and leakage of the cerebrospinal fluid in the fetal compartment. The occurrence of such congenital malformation is 1 in every 2,900 live births in the US thus making it one of the most common congenital birth defects. The failure to close the neural tube often results in the exposure of the fetal neural tissue to the external environment like the amniotic fluid resulting in intra uterine traumas during the different phases of development. Other reasons can be also attributed to this neural tube defect like the abnormality of the neural placode, exposure to drugs, toxins and genetic abnormalities [166].

Twin to twin transfusion syndrome also denoted as TTTS is observed in around 10–15 % of all monochromic pregnancies and is often related to morbidity and multiple anastomoses in the fetal circulation. This syndrome is characterized by the presence of polyhydramnios in the donor twin and oligohydramnios in the sac of the recipient twin during a monochromic pregnancy. Patho physiological conditions like early myocardiac dysfunction in the recipient twin, placental insufficiency in the donor to recipient twin are noticed [178]. TTTS if not treated can result in a high mortality rate of around 80 % and risk of disability in 20 % [179]. USG imaging is used in detecting this condition in the early trimesters. Fetoscopy laser ablation techniques have been shown to be effective over the standard procedure of amnio-reduction [180] with a median survival rate of 60–70 % before 26 weeks of gestation. The risk of severe handicap is however 5 %. This rate of overall long term survival is similar in case of amnio reduction method as well; but the major drawback of amnio reduction is that the rate of handicap is extremely high at 25 %. However another study revealed less satisfactory results for laser ablation therapy followed by an amnion reduction [178].

Discussion

In the first 8–10 days post conception, the zygote undergoes transformation into a pro-embryo and with subsequent cleavage forms the morula and the blastula stage. This early stage represents the 'All or no phase" which itself means if the embryo survives the initial toxic effect of drugs it will undergo further development and if not then it will simply lose its embryonic viability. Therefore the question apparently is whether teratogenic drugs can induce congenital anomalies in the first week of gestation itself or do they have a more profound effect at the later stages of fetal development? [181]. One paper suggests that as organogenesis is initiated from the 29th day and continues till the 70th day, this is when the DNA activation of the stem cells takes place resulting in differentiation and proliferation [181]. Therefore even if a teratogenic drug is consumed during this nascent and early period of pre embryonal development it might not have severe teratogenic implications on the embryo. Therefore considering the first trimester as a whole is a seriously challenged concept and it is

only in the second and third month when the fate of the embryo is already decided, that the fetus is highly susceptible to congenital abnormalities [181].

The use of stem cell therapies in utero is also a very exciting emerging field. Attempts have already been undertaken to treat diseases like sickle cell anemia with in utero transplantation of hematopoietic stem cells [182]. It has proved to be successful in case of allogeneic donors also. Animal studies have revealed the involvement of the immune barrier in the fetus to be stronger than it was originally supposed [183].

An attractive source of stem cells is the amniotic fluid [184]. Amniotic fluid consists of fetal origin stem cells, mesenchymal stem cells and can be easily differentiated into a variety of different lineages like myogenic, adipogenic, hepatic, neuronal and endothelial [185, 186]. Also amniotic fluid obtained from a diseased fetus through a routine clinical amniocenteses technique during the 15th week of gestation can be a viable autologus transplantation source for the ailing fetus [187, 188]. Hematopoietic stem cells harvested from umbilical cord blood collected from a first trimester fetus can be transplanted back to the fetus itself by USG guided blood sampling [189]; thereby this can act as autologus cord blood derived hematopoietic stem cell transplantation. This can also be an alternative approach as it will reduce the risk of allogeneic reaction of the fetus in its later stages of growth and development. It has been seen in mice and sheep models that amniotic fluid derived stem cells can be easily transplanted without losing any of their general characteristics [190–192].

The human immune system starts to develop between the 12th and 14th weeks of gestation and during this time there is an increase in the number of circulating T Lymphocytes in the fetus [196].

As amniotic fluid derived stem cells are fetal in origin they can show resistance against Natural Killer (NK) cells thereby playing an important role in immune suppression of the host [193]. This immune modulatory property of amniotic fluid derived stem cells is quite similar to that of mesenchymal stem cells which are regarded as one of the best immune compatible cells for transplantation. Amniotic fluid stem cells can also release high levels of cytokine factors [194]. After collection of amniotic fluid from the baby in the second trimester these cells can be easily quantified and harvested in the lab as they have a superior average doubling time of 25–38 h compared to bone marrow derived MSC's which have a doubling time of 30–90 h respectively [195]. Further these stem cells derived from the amniotic fluid have a higher clonogenic potential and can express both pluripotent and mesenchymal stem cell markers SSEA-4, Oct-4 [186].

Therefore the advantages of using amniotic fluid derived stem cells are quite paramount. After collecting amniotic fluid during the 15th week of gestation and then harvesting these stem cells and re-transplanting them back to the fetus would not result in immune rejection in the later stages of fetal development as these cells, like mesenchymal stem cells, have the ability to evade immune rejection thereby increasing the chances of enhanced engraftment in the fetus. The property of autologus amniotic membrane derived stem cell to be transplanted back to a diseased fetus in utero, can be a viable approach, limiting the number of invasive and open fetal surgeries often associated with high risk to both the mother and the fetus.

Gene therapies, like stem cell therapy, also have limitations in their approach. Gene therapy is not possible in case of thalassemia and sickle cell anemia due to the presence of the large globin gene and the globin promoter whose insertion in vectors can be technically very tough [184]. A study conducted on sheep models where autologusly derived fetal liver stem cells were isolated from a first trimester pre-immune fetal sheep with the help of USG guidance and were labeled with a PLH26 contrast and transplanted intra peritoneally into both autologus and allogeneic fetal recipients. Observations revealed equal engraftment in both the allogeneic and the autologus cases but the autologus fetal cell loss was observed to be 73 %, much higher than that of the allogeneic which was only 29 % [197].

Treatment and diagnosis of Severe combined Immunodeficiency (SCID) is feasible only in

cases of neonates. However with rapid advances in the field of in utero 2D and 3D imaging and the availability of different non invasive techniques, assessment of SCID can be confirmed even in the first trimester. Allogeneic bone marrow stem cell is the only transplantation procedure available till date through IUSCT technique [184].

In utero bone marrow derived mesenchymal stem cells have also shown long term engraftment potentialities in sheep models [198]. In case of spina bifida the therapeutic potentialities for combining surgical repair and transplantation have been shown to be effective in a rat model [199].

Apart from the scientific concepts of the fetus as a patient it is also imperative to consider the ethical view in treating the fetus as a patient. Questions arise like when does an individual human life start, rather, when does biological life begin? [200, 201]. What are its biological, ethical and religious aspects based on which we can consider the fetus as a viable or a non viable living organism? These are topics that attract a tangential amount of ideas and views. In the pre embryonic phase the zygote divides to form an embryo. During this stage of development the cells are not yet differentiated in terms of their fate for specific cells or organs [200]. By the time they reach the 14th day post conception via the formation of the morula and blastula, placentation starts and that marks the beginning of the actual embryonic phase which is accompanied along with organogenesis and morphogenesis. Therefore, it may be possible that individual human life begins with the emergence of the embryonal stage and distinct human life or a biological life starts during the initial pre-embryonic phase [200].

Any abnormalities observed during the first two trimesters of growth and development of the fetus can be rectified by means of several therapies and techniques which have already been discussed. However moral and ethical dilemmas especially in the case of open fetal surgeries still persist. The concept of open fetal surgery is a very risky option as it increases the chance of fetal and maternal mortality. Open fetus interventions become extremely necessary in cases of emergencies or for diseases that require an early intervention or might carry the risks of abortion or congenital abnormality in the later stages of gestational period. In a normal adult surgery, administration of anesthetics is not a major concern both medically and ethically, however it becomes a major issue in case of an open fetal surgery. The sole reason of anesthetics is to numb and alleviate the patients from experiencing pain while undergoing the procedure. But this concept of pain is extremely debatable in the fetus. There seems to exist different opinions among surgeons and clinicians regarding when the fetus starts to feel pain and sensation. It is suggested by many that from the 20th week onwards the fetus can start to sense some amount of pain and others claim that the receptors of pain come into prominence between the 8th and 12th weeks and the fetus can feel pain between the 26th and 28th week of its development. It is a well known fact that application of anesthetics in cases of pregnant mothers undergoing surgeries in the first and second trimester can be fatal to the fetus as there are high chances that these molecules might be able to cross the placental barrier and enter the fetal compartment. Therefore the question of moral responsibility and ethics comes into the picture when a fetus in its first or second trimester is given an anesthetic during a surgical procedure. In case of abortion the main aim is fetal destruction but in cases of surgical repair the application of anesthetics becomes highly debatable as questions of direct toxicity to the fetus arise. On the other hand if anesthetics are not administered then there are high chances that the fetus might feel pain during surgery as the feeling of fetal pain is still a debatable topic.

Conclusion

Therefore the concept of the fetus as a patient involves an extremely dynamic and diversified field of medicine and surgery which has opened doors for many new avenues and exciting ventures in the field of experimental medicine, particularly regenerative medicine, stem cell and gene therapy. The ultimate aim of treating the fetus as a patient is to maximize the chances of better fetal health and to minimize the risks associated with fetal disease

and abnormalities. The advent of pioneer biomedical imaging and instrumentation techniques will further enhance the field of pre natal diagnosis and in utero surgery.

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Structural and Functional Developmental Perspectives of the Placental Barrier and Its Role in the Fetal Development During the First and Second Trimesters

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Introduction

The term placenta has been derived from the Latin word meaning 'Cake' and the Greek word for 'flat slab like structure'. The primary function of the placenta is to act as a fetomaternal organ and a barrier with two of its components, the fetal placenta also termed as the "Chorion frondosum" and the maternal placenta termed as "Decidua basalis" [1, 2]. The fetal placenta develops from the blastocyst and the maternal placenta from the maternal uterine tissues. The first concept regarding the presence of a fetomaternal or a placental barrier was coined around 46 years ago by using transport physiology and ultrastructural analysis [3]. However in terms of evolutionary trend it is observed that the human fetomaternal barrier which is 10 nm in size is less selective than the epitheliochorial barriers in animals. The metabolic capacity of the trophoblast and macrophage along with the fetal blood cells plays an important role in the fetomaternal barrier in the first trimester and the early stages of second trimester by metabolizing and converting harmful toxins into less harmful substrates and by also forming a sequential barrier to the maternal activated immune cells [4]. Also the placenta plays a very important role in providing a micro niche which supports the fetal growth through transfer of nutrients and immunological

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properties which helps in imparting protective immunological properties to the fetus. It also acts as a major fetomaternal barrier against pathogens and the maternal immune system. The placenta also undertakes the secretion of different hormones, cytokines, growth factors and other bioactive products essential for the fetus.

Development of the Embryo

Pre Lacunar Stage

After the formation of a two cell stage, there are a series of divisions resulting in a process known as cleavage. This cleavage produces a three cell stage from a two cell stage, a four cell stage and a five cell stage. This subsequent cleavage of the ovum results in the formation of 16 cells where it looks like a mulberry thereby attaining its name as the morula. The morula remains surrounded by the zona pellucida and contains an inner cell mass surrounded completely by an outer layer of cells which gives rise to the trophoblast. This trophoblast provides nutrition to the developing embryo and forms the trophoectoderm and gives rise to the placenta in later stages of development. The inner cell mass gives rise to the embryo proper. The amnion is derived from the trophoblast layer and the chorion from the epiblast after 8 days of fertilization. Both the amnion and chorion are derivatives of the embryo [5]. With subsequent increase in the quantity of the fluid the morula takes the appearance of a cyst like structure. The trophoblastic cells attain a more flat shaped like structure and the inner cell mass gets attached to the inner side of the trophoblastic layer on one side only. This is known as the embryonic or the Animal pole and the opposite side is known as the aembryonic pole. This stage of development is often regarded as the blastocyst stage [6].

Lacunar Stage

On the eighth day of conception, the implantation of this blastocyst in the endometrium walls of the uterus commences the development of the placenta. The trophoblast layer of the blastocyst stage gives rise to the chorion and the inner cell mass later on develops to form the umbilical cord and the amnion. After adhering to the inner walls of the uterine endometrium the trophoblast layer of the blastocyst invades the endometrial epithelium, thereby eroding the deciduas and allowing the embedment of the blastocyst, a biological process often termed as the implantation stage [7]. After the implantation process the trophoblastic cells undergo rapid cell division to form a bi layered trophoblast which is composed of multinucleated outer bi layered syncytiotrophoblast and an inner mononucleated cytotrophoblast layer [7]. Appearance of small intra syncytial vacuoles in the syncytiotrophoblast starts to develop and by day 8 it becomes confluent and gets separated by the presence of lamellae and trabecule [7]. Primary villi are formed after the invasion of the cytotrophoblast into the trabeculae whereas the lacunaes form the intervillous spaces where there is a rich flow of maternal blood. This also marks the beginning of the villous stages of placentation [7].

In the first trimester of early pregnancy the anti-implantation pole gives rise to the smooth chorion due to the degradation of the villous and fibroid deposition whereas at the implantation pole appearance of leafy chorion frondosum occurs [7]. After 8–9 days of fertilization morphological changes occur in the inner cell mass forming the epiblast and the hypoblast. The epiblast constitutes the amniotic epithelium from where the three germ layers namely the endoderm, mesoderm and the ectoderm occurs whereas from the hypoblast, cells migrate to form the exocoelomic membrane which modifies to form the yolk sac [7]. This exocoelomic membrane along with the blastocoel also forms the extra embryonic reticulum. Further, some more cells from the hypoblast migrate to form the extra embryonic mesoderm which surrounds the yolk sac and the amniotic cavity and later on forms the amniotic and chorionic mesoderm. This whole process occurs before gastrulation initiates [7].

Development of the Fetomaternal Barrier

The syncytiotrophoblast layer is a continuous layer which extends all over the surface of the villi. Physiological evidence suggests that there

is an existence of two different routes through the fetomaternal barrier, namely the transcellular and the paracellular routes [8].

In the presence of tertiary villi there is an establishment of the intraplacental fetal circulation where the maternal and the fetal blood in the intervillous space comes into close contact with one another but remains separated due to the presence of the fetomaternal barrier also termed as the placental barrier which is further composed of five layers. The first layer is a continuous syncytiotrophoblast layer covering the villi and thus lining the intervillous spaces. The second layer is the cytotrophoblast layer which is consistent in its appearance in the first trimester but later discontinuous in the second trimester [8]. A trophoblastic laminal layer and the connective tissue layer which is derived from the extraembryonic mesoderm is the next layer. The last layer is referred to as the fetal endothelium layer which remains uncovered during the first and the second trimester. After the second month of pregnancy and with increase in gestational time and trimesters this fetomaternal barrier undergoes quantitative changes rather than qualitative like the thickness of the syncytiotrophoblast layer decreases from 15 μm to a mere 4.1 μm in the third trimester. Also rapid expansion of the villous surface occurs as the cytotrophoblast becomes rarified [9].

Comparison of the Fetomaternal Barrier during the First and Second Trimesters of Development

The placental barrier is composed of structures which separate the maternal and the fetal blood from one another. In the first trimester the placental barrier consists of the cytotrophoblast, syncytiotrophoblast and the villus mesenchymal layer which inhibits the entry of macrophages. During the second trimester the cytotrophoblast layer disappears from the villus wall as the thickness of the barrier subsequently decreases with increase in the vascularization and the surface area. By the beginning to mid of fifth month, i.e., in the second trimester there is a considerable increase in the number of fetal vessels along their numerous branching and sub branching which gets closer to the villous surface [10]. By the end of the second trimester the nuclei of the syncytiotrophoblast forms proliferative notes which is due to the aggregation and grouping of the cells together. The other regions of the syncytiotrophoblast lack nuclei and comes into peripheral and close proximity to the exchange zones of the capillaries thereby enhancing the rate of exchange between the maternal and the fetal compartments [10].

Also the placenta in the first trimester consists of the chorionic plate, intervillous space surrounding the chorionic villi, cell islands, basal-plate from which the septa protrudes into the anchoring villi [10]. These anchoring villi further remain connected to the basal plate via the cell columns. In the beginning of the second trimester these intervillous spaces remain filled with maternal blood. The chorionic plate in the first trimester is usually devoid of the amnion and it remains covered by a conspicuous incomplete layer of mesothelium and a thick layer of chorionic mesoderm where the chorionic vessels generally remain embedded [11]. In the intervillous space with the progression of pregnancy and gestation time the syncytiotrophoblast is replaced by the acellular eosinophilic fibrinoid [12].

At the end of the fourth week all of the placental villi become the tertiary villi and they adapt themselves according to the needs of the growing embryo. At the beginning of the eighth week there is development of the mesenchymal villi which show a homogenous, rather, a dense cellular stroma. These villi remain immature even during the second trimester and are known as the intermediate immature villi [13]. The cell islands in the first trimester are generally accumulations of the extravillous trophoblastic cells embedded in fibrinoid generally, whereas the placental septa resembles the rough pillar shape like extensions of the basal plate which protrudes into the intervillous spaces [13]. The basal plate which is also the bottom part of the intervillous space represents the part of the fetomaternal junctional zone which remains adhered to the placenta after delivery [13].

The placenta in the second trimester generally has a similar structure to that of the placental development as observed in the first trimester with the exception that in this stage the layers are much better defined [10]. In the second trimester the cytotrophoblast slowly disappears from the tertiary villi walls and thereby diminishes the

distance between the maternal and the fetal circulation. These cytotrophoblasts also disappear from the chorionic plate whereas in the basal plate the cytrophoblast layer still persists [10]. Along with the deciduous formation and the deposition of fibrin, protrusions are formed which project into the intervillous spaces and thereby form the cotyledons which are normally primitive in appearance during this time. In the meantime septas are formed due to the spread of the placenta [14].

Villous Development in the First and Second Trimesters

On the 12th day after conception the transition phase starts where the implanted embryo moves from the lacunar phase to the primary villous, accompanied by secondary and tertiary villous formation stages, and this continues till the 18th day to term [8]. In the tertiary villi as soon as the intra placental fetal circulation develops, the fetal blood in the villi and the maternal blood in the intervillous come into close contact with each other but remain separated by the placental barrier [8]. This placental barrier is made up of a continuous syncytiotrophoblast layer, an initially complete cytotrophoblast layer in the first trimester. However this becomes inconsistent in the second and third trimester period. With increase in the trimester period there are extensive structural and functional changes in the blood placental barrier like the thickness of the syncytiotrophoblast layer decreases; the cytotrophoblast becomes relatively rarified due to the constant increase in the villous surface areas [8]. The intervillous position of the fetal capillaries comes closer to the villous surface with advancements in maturation resulting in the reduction of mean maternofetal diffusion distance [8]. This distance decreases from 50 to 10 μm and further between 4 and 5 μm at term gestation [15]. The exchange between the fetus and the mother is focused on the presence of terminal and matured villi which remain supplied by fetal capillaries lined by continuous epithelium without any pores [15]. Its main function is to act as a passive filter and limit the transfer of molecules with larger weight, just like in the blood brain barrier, the presence of pericytes and smooth muscle cells also helps in the control of permeability and vaso regulation [16].

The synctiotrophoblast separates the mixing of maternal and fetal blood. Generally it is continuous and uninterrupted and extends all over the villous trees. The transplacental exchange mainly takes place by the paracellular and the transcellular route. The paracellular route of exchange is mediated via the extracellular water filled pathways and the transcellular involves the bi lipid plasma membrane layer and the cytoplasm of the synctiotrophoblast. Occurrence of blood clots and exchange of toxic substances prematurely can be attributed to the fact that the syncytiotrophoblast does not always maintain an intact integrity [8].

Blood Circulation across the Placenta during the First and Second Trimesters

Maternal Vascularization and Placental Circulation

Maternal vascularization is often defined as an important step in the pre implantation stage where the structural changes of the spiral arteries occur resulting in the disorganization of the smooth muscle cell layers and the matrix by means of proteinases such as MMPs [17] along with vasodilation and a reduction in blood pressure. This further assists in increased flow of maternal blood towards the placental surface due to the decrease of maternal arterial resistance [18–20]. The maternal blood placental circulation is matured and completed by the end of the first trimester. During the pre implantation of the blastocyst stage, the walls of the uterine endometrium epithelial cells undergoes morphological changes also defined as “decidualisation.” The spiral arteries are remodeled and there is an increase in the diameter size of these arteries [21]. They also become less convoluted. The increase in the diameter helps in the increased pressure of blood flow from the maternal end to the intervillous space of the placenta thereby bathing the fetal villi. In the case of human placentas the maternal blood comes into direct contact with the fetal chorion although there is no exchange of fluid with the fetal chorion. Further due to a decrease

in the pressure, the deoxygenated blood flows back through the endometrial veins.

Fetoplacental Circulation

On the other hand deoxygenated blood from the fetus is passed though the umbilical arteries to the placenta. These umbilical arteries radiate to form chorionic arteries at the junction of the umbilical cord and the placenta and further divides to form an extensive network of arterio capillary venous branches in the villi brining the fetal blood in close proximity to the maternal blood but with no mixing due to the presence of the placental barrier [8]. Vasoconstriction is caused by endothelin and prostanoids in placental arteries whereas nitric oxides cause vasodilation. Free radicals may be generated as the feto placental circulation is extremely sensitive to hypoxia resulting in preeclampsia [22]. Melanin can play a major role in such cases by acting as an anti-oxidant [22]. Also certain other molecules like oxygen, water, electrolytes, carbohydrates, lipids, proteins, amino acids, vitamins, hormone, drugs including pathogens and viruses can enter the fetal compartment during the first trimester from the rich maternal spiral arteries. On the other hand in the fetomaternal exchange system, carbon dioxide, water and urea along with any waste products or hormones can enter the uterine circulatory system from the fetus. During the course of development the maternal and the fetal circulatory exchanges comes into close contact without any mixing due to the presence of a thin layer of syncytiotrophoblast. Any disruption in the flow of blood can eventually hamper the fetomaternal circulatory system thus causing hypoxia to the fetus during the first and the second trimester [8, 23, 24].

Physiological Properties of the Placenta during the First and Second Trimesters

The placenta consists of Umbilical vessels, chorionic plate arteries and multiple dividing villi. It lacks any form of autonomic regulation and instead, the vascular function is under the direct influence of the hormones [25] thereby stimulating the roles of agonists in the placenta function. There is a delicate balance between the role of vaso constrictors and vaso dilators in controlling the placental circulation. Presence of mechanosensors in the placenta can be a possibility as it has been shown to adapt to various hemodynamic changes [26]. Another important property of the placental circulation is that it depends on the adequate matching of the placental and uterine blood flow and has shown to have affinity towards Angiotensin II receptors and ACE [27–29]. Other potent vasoconstrictors include Et-1 released from the endothelial cells and are found abundantly across the placenta and umbilical vessels [30–33], thrombaxanes and 5-HT [33, 34].

Role of Vasculogenesis and Angiogenesis in the First and Second Trimester Development of the Placenta

Angiogenesis starts from day 32 and proceeds till week 25 after conception. During week 15 when a regression of peripheral capillary nets takes place, the remaining central capillaries develop into primitive veins and arteries. Due to vascularization of the maternal spiral arteries the maternal adaptions to pregnancy are almost complete by the end of the first trimester during which the maternal blood via the developing fetomaternal passage enters and floods the placental intervillous spaces but does not mix with the fetal blood. This begins at the eighth to ninth week and it increases more dynamically by the time it reaches the end of the first trimester, i.e., the 12th week [35–38].

Presence of Vascular endothelial growth factor (VEGF) and Placental growth (PGF) factors in the maternal plasma normally remains high at around 25 days post conception and this trend continues till the second trimester [39, 40]. In the presence of angiogenesis and a relatively hypoxic environment, the trophoblast cells increase their proliferation to form the highly vascularized villi and eventually a complex network of blood vessels which begin to transport blood [19, 41, 42, 44]. Due to further enhancement of the level of angiogenic factors, there is an increase in the

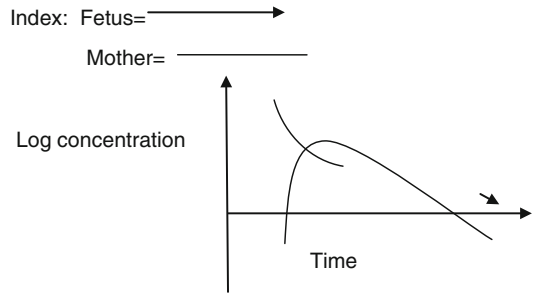
formation of blood vessels from pre existing ones and eventually develops into well defined arteries, veins and arterioles [43]. Therefore the exchange of nutrients, gases, oxygenated and deoxygenated blood initiates at this stage [37]. The placenta helps in a stable passage of blood continuously through its vessels due to its low resistance system [27] and a pressure difference between the vascular connections which further drives the blood from the basal part towards the chorionic plate [19, 35, 42, 44]. The function of the early or primary villous trees are incomplete at this stage as they fail to develop into a fully functional system lacking autonomic control to help keep the fetus constantly supplied with blood. In the second trimester no new vessels are formed rather sprouting of multiple villi branches takes place which are constantly replaced by immediate villi for the rest of the trimesters [8, 25].

Mechanisms of Fetomaternal Exchange

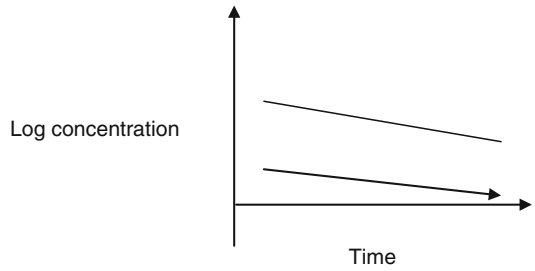
There are different types of mechanisms by which feto maternal exchange takes place. They are either simple diffusion by which mainly gaseous substances like oxygen, carbon dioxide and fatty acids gets exchanged. Active transport includes the passage of Iron, Calcium and Iodine whereas facilitated diffusion includes the passage for glucose. Amino acids can pass through secondary active transport mechanisms whereas electrolytes and water are exchanged via bulk transport system. However taking advantage of this nature's unique system of selective exchange between the mother and the fetus, often harmful substances like nicotine, cocaine, alcohol, sedatives, anesthetics and analgesics can indulge in maternal fetal exchange causing inhibiting effects on the fetus.

The law by which fetomaternal exchange is governed is as follows: $Q/T = K \times A(Cm\text{-}Ct)/D$ where Q/T is defined as the rate of diffusion, K is the diffusion co-efficient, A is the area of the membrane, D is the thickness of the membrane and (Cm-Ct) is the concentration gradient. This is also known as Fick's Law [8].

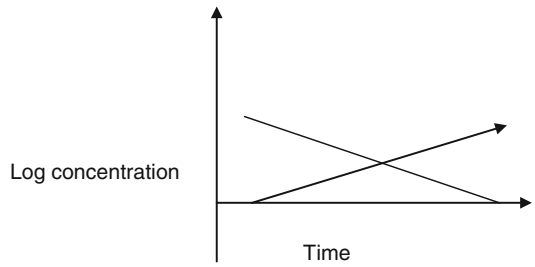
Pharmacokinetics of Maternal and Fetal Exchange of Drugs



The above graph depicts when the rate of placental transfer is high. The drug crosses the placenta and reaches the fetus rapidly. The curve overlaps and reaches an equilibrium state because the maternal and fetal plasma protein binding level rapidly rises and reaches concentration equilibrium with each other over time.



This type of graph is obtained when there is no feto maternal exchange. This not possible practically as in reality it does shows some varying degree of exchange. Several reasons like the fetal metabolic status, plasma protein binding capacity, and its excretory function may be cited.



The graph above depicts a case where the maternal fetal exchange is rapid across the placenta. With increase in time fetal concentration of drug and protein may increase suggesting that the fetal concentration of drugs might be higher than that of the mother. This can be attributed to the presence of drugs in the maternal system and lack of maternal renal clearance or in the fetus because of the lack of a developed renal clearance function or hepatic metabolism due to which the fetal drug concentration increases over time.

The placenta favors the entry of lipophilic molecules. Processes like endocytosis, exocytosis, pinocytosis and the free movement of vesicular vehicles also contribute towards the fetomaternal exchange. Low density lipoprotein, transferrin-iron complex, IgG all enters the placenta by mechanisms of endocytosis and exocytosis [45]. Uptake of IgG protein is by a receptor mediated endocytosis and it is transported in vesicles so as to negate the effect of phagolysosomal degradation before it enters the fetus.

Hydrophilic permeability of the lipid insoluble molecules in the absence of transporters is extremely difficult to cross the placental barriers. However presence of extracellular pores can facilitate this transfer of such molecules due to the fact that the surface area is quite small. This phenomenon is termed as Membrane Limited [46–48]. Permeability of hydrophilic substances is also dependant on the diffusion co-efficient of water. Presence of para cellular channels and pores are also sufficient enough to allow the free diffusion of low molecular weight inorganic substances like chloride, phosphate and sulphate ions. These inorganic ionic concentrations are seen to be in a higher concentration in the maternal plasma thereby providing an evidence for transcellular active transport mechanism.

Also it has been seen that stereo-specificity plays a major role in the materno-fetal transfer not only in the case of transfer of amino acids but also in cases of some drugs which might explain the unpredictability that why some drugs in spite of being lipophilic and non ionized find it difficult to cross the placenta. In case of an anthelmintic drug Albendazole Sulfoxide which was administered as a single dose to a sheep during the last trimester, it was found that there was a higher impairment of Albendazole Sulfoxide (+) transfer from the mother to the fetus unlike the Albendazole Sulfoxide (−) [49, 50]. In another a chiral drug, NSAID Ketotifen, which can cross the placenta readily has shown that the S(+) Ketotifen due to its stereoselective protein binding can increasingly cross the placenta unlike R(−) Ketotifen thereby indicating the great variability of transplacental transfer even among the same drugs based on their streochemical properties [51].

Role of Efflux Transporters and Their Level of Expression in the Fetomaternal Barrier during the First and Second Trimesters

In the course of pregnancy the expression of the different active transporters in the placental cellular structure evolves and some of them are found to dominate in the first trimester and others in the second trimester. Still others are expressed continuously throughout the gestational period [52, 53]. High levels of P-gp are expressed and detected on the human and mouse syncytiotrophoblast layer forming the essential part of the hemochorial placental barrier [54–59]. This presence of efflux transporters in the placental barrier plays an important role in the placental resistance in the passage and exchange of drugs and proteins during the first and second trimesters of development. P-glycoprotein (encoded by the ABCB1 gene) and a member of the active binding cassette (ABC) group are found to be expressed on both the maternal and the fetal sides although expression on the maternal villi is more [57].

However as mentioned above, the expression of the various placental transporters varies at different gestational periods and during each trimester of pregnancy [53, 60, 61]. The expression of P-gp efflux transporter protein is observed to be the highest in the first trimester, up to 45-folds higher when compared to the second and third trimesters and it decreases subsequently with increase in gestational time [56], thus suggesting

the protective role of P-gp during the early trimester phases and its direct importance in providing a protective mechanism in the early pregnancy stages when the fetus is at its most vulnerable stage due to the occurrence of organogenesis [52]. However expression of an organic anion transporter like OATP2B1 and the breast cancer resistance protein BCRP are more or less consistent throughout the first and second trimesters without any noticeable changes in their expression levels [52]. To compensate for the loss of P-gp expression in the subsequent second trimester it is thought that other efflux transporters such as the Multi drug resistance associated protein 2 (MRP 2) plays an important role as their level of expression increases more towards the end of second trimester [52, 62, 63].

During the first trimester period, the epithelium remains stratified and the thickness of the fetomaternal barrier often exceeds more than 20–30 μm [64]. With increase in gestational time and as the weight of the fetus increases the placental barrier undergoes thinning with most noticeable changes observed in the late second trimester phase and continuing till term pregnancy. The size of the fetomaternal barrier gets reduced to 2–5 μm from 20 to 30 μm in the first trimester [64]. The reduction in barrier thickness can be attributed to a number of factors in terms of change in morphology and structure like the extension of the chorionic villi capillaries, atrophy and reduction of the continuous arrangement of the cytotrophoblast cells and transformations in the poly nuclear structure of the syncytiotrophoblast. These changes in the fetomaternal barrier have a direct consequence on the transport ability of the drugs and the role of the placenta as a drug metabolizing organ in the later stages of development [65].

The above changes in the placental structure play an important role in affecting the expression of the various ABC and other organic and anionic transporter families. The barrier role of the placenta undergoes subsequent evolutionary and adaptive changes, i.e., from the importance of its protective function to the embryo to the free exchange of selective substances between the mother and the fetus by the end of second trimester [64, 66]. However in the first 10 weeks of the first trimester due to extensive and intensive organogenesis there is a lack of extensive fetomaternal circulation as observed in the case of the second trimester. This does not helps in complete elimination of embryo toxicity and its effects with respect to xenobiotics as some of them depending on their small size can still enter the fetal compartment by ways of bodily fluid tissue via the intercellular spaces [65].

Multi Drug resistance protein 3, whose transportation is still not well documented, has shown to express on the baso-lateral segments of the syncytiotrophoblast plasmalemma and in the villi endothelial cells of the capillaries. It has shown to increase with gestational time with the first trimester showing the lowest expression compared to the second trimester.

Other important transporter members of multi drug resistance are the MDR 1 and 2 efflux proteins. These have also shown to increase their expression with increase in the gestational phase [67]. MRP 5 another sub class of the MRP family has shown its activity to be high during the first trimester as it helps in the regulation of organogenesis and differentiation of the fetus. MRP 7 another member is found in low concentration at the placenta as its cellular localization is yet to be established. Their main role is to help in resisting the entry of anti viral drugs and effluxing cyclic nucleotides, bile acids, conjugated steroids and eicosanoids [65].

Therefore it can be concluded that the functions of the above discussed transporters in the placenta is still not established as also their role in inferring resistance to the placental barrier for the exchange and passage of substances between the maternal and the fetal compartment.

Role of Placenta as an Immunological Barrier during the First and Second Trimesters

Medawar and Billingham during 1953 proposed a hypothesis in a *Nature* article to explain the idea why the fetus is not rejected as a graft by the mother [68]. According to them either the con-

ceptus lacks immunogenicity or there is a significant lowering of the immune response during the onset of pregnancy or the uterus is an immune privileged site like the brain. They also postulated that the presence of a placental barrier might regulate the immune components selectively. After studies were conducted in mouse models by Hoskin and Murgita it was revealed that there were immune reactions against the fetal mouse thereby refuting the theory of lack of immunogenicity in the conceptus [69]. Also the occurrence of ectopic pregnancy further disapproved the claims that the uterus might be an immune privileged organ site. Therefore the remaining two hypotheses, i.e., whether the maternal immune system gets lowered during pregnancy and the presence of the placental barrier controls the immune response were tested and remain under intense scrutiny [70]. Cytokines have also been shown to play an important role in regulating embryo survival apart from maintaining the angiogenesis and vasculogenesis in the initial stages of pregnancy. Due to the production of huge cytokines from the uterine decidual and placental cells an immune response deviation occurs from Th1 to Th2. Although this leaves the mother at of high risk of infection but on the other hand it imparts stability and maintenance of pregnancy by controlling the endocrine system thus promoting the function of trophoblast at the implantation site [71]. The fetal trophoblast plays the major role in evading recognition by the maternal immune system. Formation of the placenta, the organ that feeds the fetus, involves cooperation between maternal NK cells and fetal trophoblast cells that remodels the blood supply. This process and, consequently, human reproductive success, are influenced by polymorphic human leukocyte antigen (HLA)-C ligands and their killer cell immunoglobulin-like receptors [72]. HLA plays a crucial role in the process of implantation [73]. During the time of embryonal development in the first trimester half of the fetus receives fetal antigen from the mother and the rest is from the paternal antigen. Due to this characteristic over expression of HLA-G is found on the trophoblast whereas expression of the HLA-A and B are absent and HLA-C is weakly expressed during the first trimester [74]. HLA-G has shown to bind to KIRs (killer cell like Ig-like receptors of NK cells) and block the cells' toxicity and infer tolerance to the fetus by reducing the NK cells activity [70]. LIF also synthesized in the first trimester by the deciduas on the maternal part of the placenta plays an important role by binding to LIF receptors; this further helps in trophoblastic growth and differentiation thereby providing fetomaternal tolerance and acting as an immune barrier [75].

In fetuses of the second trimester of pregnancy, the Igs, SC and J chain are located in the syncytio- and cytotrophoblast of the chorion [76]. The villous stroma contains a small amount of different subsets of lymphocytes. Macrophages account for up to 45 % of the stromal cells of the villi and contain IgG and J-chain. In the maternal part of the placenta, the Secretory Immune System proteins (SIS) are in the decidual cells. Relatively few lymphocytes and macrophages are observed in the decidual stroma. A different origin and composition of immunocompetent cells and a different course of immune reactions in fetal and maternal parts of the placenta have been shown [77]. These systems are already in place at the beginning of the embryonic period starting from weeks 3.5–4 to 5 and function during the entire first trimester of pregnancy.

The trophoblastic villi has several protective mechanisms for the fetus in place against the maternal system, for instance, it fails to express MHC class I and II molecules and down regulates maternal NK cells by the expression of non-classical MHC gene [78–81]. Also expression of the complement proteins like CD46, CD55 and CD59 by the surface of the trophoblast helps in protecting the embryo in the first trimester [82, 83].

The Secretory Immune System in the Barrier Structures during the First and Second Trimesters

In the mucosal and systemic compartments of an organism after an antigen expression, the secretory immune system (SIS) participates and

helps in imparting immune protection of the organs. Two types of the SIS have been suggested to be present at the border between maternal and embryonic tissues [76, 84]. Therefore it acts like a barrier between the external and the internal environment for the organ [85, 86]. The SIS expressed on the maternal placenta [76] consists of an integrated extensive pathway of lymphoid tissues which are made up of inductive and effector sites for the protection of the host organ against the foreign pathogens/antigens [87]. The fetal part of the placenta also comprises of IgG, IgA, IgM along with trans membrane secretory components (SC), macrophages, J chain, antigen presenting cells and plasma cells [82, 85, 86, 88]. Secretory components are classified as glycoprotein components and are an important receptor site for SIS [78, 89]. In addition to the mucous membranes and glands of the human embryos and fetuses, secretory cells (SC) are also detected in the trophoblast and amnion. During the first trimester at around the fourth week, presence of SC and J chains in an up regulated state are detected in the absence of lymphoid organs in the ectodermal and endodermal structures of the embryo thus helping in functioning as a pathological barrier against the entry of foreign pathogens to the fetal compartment [82]. During this 3–4 weeks time and also during the onset of the second trimester the fetomaternal part of the placenta starts to express all types of components for the secretory immune system SIS [76]. Although SIS is fully expressed during the third trimester of gestation, in the second trimester itself the placenta expresses all the typical components of SIS [90].

Therefore the fetomaternal barrier function can be very much related to that of the mucosal SIS although some exceptions like secretion of IgG at a higher level occurs in the placenta and IgA and IgM to a much lesser extent partly due to the fact that IgG helps in providing the fetal immunity primarily. Unlike the mucosal SIS the placental barrier in the first and second trimesters, does not possess a large surface area [91].

Role of Epigenetic Factors in the Formation of Placenta from the Early Gestation Phase till the First Trimester

The placenta through its blood placental barrier protects the fetus from the maternal immune system. It attacks and helps in the detoxification of harmful drugs and in the secretion of growth factors and pregnancy associated hormones. A major factor in the placental growth and development is its genetic imprintation and how it is controlled [92]. After fertilization, there is a genome wide DNA methylation and remethylation observed in the embryos during the process of blastocyst formation in the first trimester. There is a lack of DNA methylation in the Tropho Ectoderm however [93]. It is also observed that the level of DNA methylation is extremely active in the embryonal formation period rather than the placentation process [93]. In many ways the development of the placenta is related to the properties of tumorogenesis like trophoblast proliferation and migration, invasion of the uterine endometrial walls and escape immune regulation, thereby conferring the fetus an immunological privilege [94]. These all are controlled by DNA methylation and histone modifications of tumor suppressor genes such as Maspin, RASSFIA and APC in human placentas [95]. Novakovic et al. in 2008 also demonstrated through their investigation that during the first trimester there is a methylation dependant reduction in the expression of a small subset of genes during the normal placenta formation process [96]. Also pluripotency of the inner cell mass is maintained by the Histone 3 arginine methylation of the blastomeres [97]. Mutants of the polycomb group family fail to form the chorion and the amnion of the placenta [98, 99].

Is the placenta an important site for epigenetic action? It has been shown that the placenta fails to develop in the first trimester in the absence of Ascl2, Phlda2 and Peg10 genes. Igf2 gene has been shown to play an important role in the development of the selective diffusional placental

capacity thereby raising the inevitable question regarding whether the placenta is a hub for epigenetic actions or not [100–105].

Therefore it can be said that epigenetics which is a relatively new field is gradually getting recognition as an important diagnostic and developmental study tool around the globe for understanding the different transcription factors which are responsible for placental development especially during the first two trimesters as the maximum development of the placenta is associated with those two stages. The epigenetic regulation is a highly dynamic process that starts from the first trimester and continues till fetal development and includes susceptibility in later life [94].

Discussion

The placental barrier which is a highly specialized organ containing the maternal and fetal parts, acts as the main structure providing normal function and development to the fetus [78]. The growth and development of the placenta in the first two trimesters are highly coordinated and regulated by the different transcription factors at the molecular level. The formation of the placental barrier and its intactness in the first two trimesters is meant for selective exchange of substances like removal of carbon dioxide from the fetus and introduction of oxygen through the maternal blood circulation. Glucose, another important molecule, can pass freely via the pores of the syncytiotrophoblast layer via GLUT 1 [106] and during moderate glucose deprivation through the GLUT 3 transport protein [107]. However severe glucose deprivation can have an adverse effect on fetal growth and development.

Another role of the placenta in the first two trimesters is to help in metabolizing harmful substrates that might cross the feto maternal barrier thereby making entry into the fetal compartment [64]. The placenta helps in providing protective mechanisms against entry of xenobiotics, different pathogens and proteins that might cause harm to the fetus during the first and second trimester as layout of organogenesis is initiated during the initial phases of the first trimester. The placenta also primarily eliminates fetal acid equivalents of respiratory and metabolic origin from the fetus. Passage of IgG protein molecules is initiated even in the presence of strict placental barrier from the first trimester in order to compensate the immature fetal immunity. This transport of IgG is initiated between week numbers 4 and 5 of post conception in the first trimester phase [84]. It has also been claimed that only the IgG molecule can pass across the placenta into the fetus and not other Ig's [108, 109]. However in case of Toxoplasma Gondii, an infectious disease, it has been seen that IgA can also transfer from the mother to the fetus [109, 110]. This once again highlights the very true nature of the fetomaternal barrier which allows the exchange and passage of selective molecules just like the blood brain barrier in the adult system. However any inflammation in the placenta can result in the hyper presence of Ig's in the fetal compartment as the transport of all Ig's will increase respectively. By the time the ninth week is reached, fetuses are able to synthesize their own IgG sub class 1 and 2 [111]. Of late, new findings have emerged on the fetomaternal exchange of fetal stem cells between the mother and the fetus. This phenomenon is known as microchimerism where fetal cells persist in the maternal circulatory system and tissues, playing a major role in healing with response to injury. One such case was reported in a mother suffering from Hepatitis C where the fetal cells aided the maternal system to control her disease. On the other hand maternal chimerism can result in the transmission of cancer, dermatological and systemic auto-immune diseases to children [5].

Conclusion

The placenta functions as a fetomaternal barrier which remains highly selective during the first trimester. With subsequent decrease in the fetomaternal barrier the selectivity also decreases by the end of the second trimester. Also the physical and anatomical appearances of the placental barrier throughout the first and

second trimesters are synchronized with fetal development, thereby proving the important point that fetal growth and development during these first two trimesters essentially rely on the presence of the placental barrier, since abnormalities in the placental structure can disrupt the integrity of the fetomaternal barrier leading to abortions or other fetal adverse events in the first and second trimesters.

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Part XIV

Surgical Implications of Fetal Development: Up to Second Trimester

Surgery of the Unborn/Fetal Surgery

36

Mursheed Ali, Priyodorshi Sengupta, and Niranjan Bhattacharya

Introduction

Fetal surgery is part of the expansive scope of surgical systems that are utilized to treat birth defects in the fetal stage. Fetal surgery has improved from a health curiosity to a multidisciplinary specialty for treating various diseases and to improve patient outcomes. There are two predominant types of fetal surgery techniques: one is the open fetal surgery method in which the uterus is opened for operating the fetus; the other technique is fetoscopic surgery, in which incision is minimal. The techniques adopted for this surgery are sonography and fetoscopy. In initial stages of fetal surgery during 1990s only folic acid supplements were prescribed to women considering high risk, which was borne previously with a child by NTD. But, the recent advancements in the fetal surgery techniques allow the physicians to diagnose abnormality of the fetus accurately and to treat fetal anomalies with high level maternal safety. Therefore, neonatologist needs to be more familiar with recent techniques which are shaping the field. The purpose of the present review is to examine emerging operative treatments for fetal abnormalities including percutaneous, fetoscopy and open hysterotomy methods that are used for fetal surgery. The effectiveness of these experimental therapies is also analyzed.

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Emergence of the Field of Fetal Surgery

In 1963, Liley, a New Zealand perinatologist, successfully initiated the new area of fetal surgery by transfusing blood into the peritoneum of a hydropic fetus affected with severe Rh disease [1]. The complete transfusion, yet, involves straight access to the fetal circulation by hysterectomy [2] or rod lens fetoscopic surgery [3]. A group of surgeons in Nashville during 1999 operated the spina bifida. They performed the surgery for a 147 days old fetus in the womb. The operation was performed by a surgical team who developed a fetal correcting technique in mid-pregnancy by removing the uterus temporarily, then draining it of amniotic fluid, performing a tiny operation, and finally restoring the fetus to the uterus [4].

The first clinical fetoscopic surgeries were done through obstetrical endoscopy, where the outcome of a clinical trial demonstrating fetoscopic laser

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coagulation for chorionic plate vessels proved effective for Twin-to-Twin Transfusion Syndrome (TTTS). At present for severe congenital diaphragmatic hernia, fetal tracheal occlusion surgery has been performed and been proved safe. Minimal access fetal surgery is important in preventing preterm labor which can occur with invasive surgery; it is risky for the mother because there is a risk of preterm prelabour rupture of the membrane (PPROM). This proved to be a bottleneck for further developments. Risto Juhana Rintala et al. in an article in 2014 highlighted the research methods by which valid evidence-based research data is obtained in observational studies, randomized controlled trials, and meta-analyses. Consequently; very few designed trials in neonatal surgical treatment have been performed [5]. Hence valid evidence-based research data are few. However, the authors suggest that the problem of small numbers of patients may be overcome by meta-analyses, multi-center trials, and networking.

Cystic Lung Lesions

Congenital Cystic Adenomatoid Malformation (CCAM) is a condition in which abnormal tissue grows as cysts in a fetus's lungs. This may require open fetal surgery if hydrops appear. Open fetal surgery is a modified type of cesarean where physicians make an incision in the uterus, exposing the fetus's chest and perform surgery to remove the cysts. The fetus is then returned to the womb and the uterus closed and the pregnancy carried out until term. CCAM fetuses who have open fetal surgery have an increased survival rate of 50 – 80 percent. However, it places the mother at a high risk for preterm labor and complications. Cases have been documented at the University of California, San Francisco, Medical Center (UCSF) and Children's Hospital of Philadelphia (CHOP). A group of researchers determined CVR (CAM volume ratio) by measuring 3 dimensions of the CAM using the formula for the volume of an ellipse and dividing by the head circumference to correct for differences in gestational age [6]. The study showed that when the CVR is greater than 1.6, the danger for hydrops is 80 %, and in such cases, fetal intervention may be necessary (thoracoamniotic shunting, fetal thoracotomy, EXIT delivery). In the appropriate situation, this maternal fetal surgery approach for CCAM is life-saving for the affected fetus with acceptable maternal morbidity risks in the present and future pregnancies [7, 8]. Hydrops can be resected in utero if they are predominantly solid or multicystic. Fetal sacrococcygeal teratoma complicated with progressive high output cardiac failure may benefit from in-utero resection of the tumor [9, 10]. At the point when introducing late in pregnancy they can be resected while on placental flow [11]. Macrocystic masses can be punctured or shunted. The Center for Fetal Diagnosis and Treatment, The Children's Hospital of Philadelphia (CHOP) has done the largest number of highly complex surgical interventions to repair birth defects in the womb, approximately 1000 out of 4,000 fetal surgeries done worldwide, by 2013. As far as they can tell, fetal intercession lessens the CCAM volume by 70 %, turns around hydrops and results in a survival rate of 74 % [12]. This rate has been affirmed by others [13]. Recently there have been studies on the utilization of steroids on the mother. Peranteau et al showed that maternal prenatal administration of Betamethasone (12.5 mg/day, im, two consecutive days) may have beneficial effects on clinical fetal evolution in cases with massive CCAM (microcystic) with or without hydrops. However, more studies are needed to prove its adequacy [14, 15].

Sacrococcygeal Teratoma

Some sacrococcygeal teratomas (SCT) do not result in pre-birth issues; larger and quickly growing tumors increase the metabolic requirements of the fetus, thereby causing fetal anemia and acting as a vast arteriovenous shunt, in the long run bringing on high-yield cardiac arrest. This prompts polyhydramnios, hydrops and intra-uterine fetal death (intra-uterine fetal demise IUFD). Moreover, the mother may develop mirror syndrome. The groups from UCSF and CHOP showed that fetal hydrops and placentomegaly are indicators of poor outcome [16, 17]. Others have proposed extra

prognostic criteria, for example, rate of tumor development taking into account which fetal treatment may be considered [17]. A study conducted by a group at the Hôpital Necker-Enfants Malades AP-HP-Université Paris V on 44 fetuses showed that in prenatally diagnosed sacrococcygeal teratoma (SCT), there is good maternal and perinatal outcome when the tumor diameter less than 10 cm, absent or mild vascularity and slow growth or when it is diameter 10 cm or greater, predominantly cystic lesion with absent or mild vascularity and slow growth although shunting or drainage of the SCT could be necessary. Large fast-growing SCT with rich vascularity is associated with a higher perinatal mortality and morbidity than smaller lesions with mild vascularity [18].

Symptomatic treatments, for example, amniodrainage (polyhydramnios), intra-uterine transfusion (anaemia) and bladder shunting (urinary obstacle) have been reported as well. Open fetal resection of Type 1 or prevalently additional pelvic tumors has been reported in five cases. Mean age at childbirth was 30 weeks and four survived [19]. One survivor had to undergo postnatal treatment of pulmonary metastases of a germ cell tumor and at age of 11 years had no confirmation of illness; however, another patient had significant morbidity, which could most likely be identified with emboli at the time of tumor resection. The other two survivors stayed healthy. There are also reports of less invasive procedures, arresting flow in vessels either by fetoscopic laser [20], interstitial thermocoagulation [21] and also radiofrequency removal [22]. The latter can result in collateral damage of the tissue [23].

A group of researchers have also suggested that in utero embolization with Histoacryl may be a useful tool to reduce cardiac output in large vascular sacrococcygeal teratoma [18, 21, 24].

Myelomeningocoele and Other Open Fetal Surgical Methodologies

Myelomeningocoele (MMC), one of the most common congenital malformations, can result in severe lifelong disabilities, including paraplegia, hydrocephalus, Arnold-Chiari II malformation, incontinence, sexual dysfunction, skeletal deformations, and mental impairment. MMC was the first nonlethal anomaly to be treated by fetal surgery. The first cases of in utero spina bifida repair were performed in 1994 with an endoscopic technique that proved unsatisfactory and was abandoned. In 1998, in utero repair of MMC was performed by hysteromy at the Vanderbilt University and at the Children's Hospital of Philadelphia (CHOP). Adzick reviewed the natural history, pathophysiology, and in-utero intervention for fMMC. The author conceded that little progress has been made in the postnatal surgical management of the child with spina bifida. Postnatal surgery is usually aimed at covering the exposed spinal cord, preventing infection, and treating hydrocephalus with a ventricular shunt. In-utero repair of open spina bifida is now performed in selected patients and presents an additional therapeutic alternative for expectant mothers carrying a fetus with fMMC. Tests and early clinical experience demonstrated that pre-birth treatment could enhance result [25]. Observational studies demonstrated that pre-birth microsurgical layered repair turns around hindbrain herniation, diminishes the requirement for shunting, enhances leg and bladder capacity, and in addition later cognitive function [26–28]. The National Institutes of Health has supported the Management of Myelomeningocoele Study (MOMS) randomized trial. The objective of the trial was to evaluate if intrauterine repair of MMC between 19 to 25 weeks gestation improves outcome compared with standard neurosurgical repair. The MOMS trial has shown a significant reduction of VP shunt placement at one year of age following fetal MMC surgery (prenatal group: 40 % vs. postnatal group: 82 %, $P<0.001$). The trial also demonstrated a substantial improvement in the overall neuromotor function at 30 months of age by a variety of measures including the finding that 42 % in the fetal surgery group were walking independently compared to only 21 % in the postnatal surgery group ($P<0.01$). Finally, hindbrain herniation was significantly reversed in the fetal surgery group compared to the postnatal surgery group (no hindbrain herniation in 36 % and 4 % of the infants, respectively, and

severe herniation in 6 % and 22 %, respectively, P < 0.001). But it also revealed that fetal MMC surgery increases the risks for spontaneous rupture of membranes (prenatal surgery: 46 % vs. postnatal surgery: 8 %, P < 0.001), oligohydramnios (21 % vs. 4 %, P = 0.001), and preterm delivery (79 % vs. 15 %, P < 0.001) including 13 % of fetal surgery group that were born before 30 weeks of gestation. This trial is intriguing for some reasons. Initially, it is being conducted only in the US. Second, the trials are helping to bring together contending opinions regarding MMS. The main downside is that it is not open to non-US subjects. Additionally such a trial may stall the advancement of negligibly intrusive methods. However, other trials have provided promising results. For instance, an experiment using in utero repair of congenital myelomeningocele through a hysterotomy appeared to be technically superior to procedures performed endoscopically [29, 30].

Open fetal surgery is substantially less popular in Europe. Most centers limit themselves to procedures performed on placental support. The EXIT (ex utero intrapartum treatment) procedure was at first designed as a delivery technique for safely establishing upper airways following tracheal occlusion [31, 32]. The specialized subtle elements of this treatment modality have been precisely detailed [11, 33], and most fetal surgical centers should be required to have the capacity to offer this treatment modality safely. The time between induction of anesthesia and cord clamping is kept as long as is clinically needed. Utilizing inhalational anesthesia for maximal uterine relaxation, uteroplacental blood stream and gas exchange are maintained and amnioinfusion and incomplete conveyance of the fetus keep uterine volume within normal limits. The list of indications for utilizing this method has developed through the years yet basically incorporates airway obstruction because of laryngeal atresia, vast tumors or iatrogenic tracheal impediment. The procedure may also be used to create vascular access for extracorporeal circulation, such as for managing cardiac defects, severe CDH, lung lesions or conjoined twins [34].

Michael Tchirikov et al. in 2012 investigated the impact of undertaking long-distance air travel to a specialized medical center while pregnant in order to undergo fetoscopic laser coagulation (FLC) for twin-to-twin transfusion syndrome (TTTS) [35]. A retrospective cohort study was conducted on a women having TITS who travelled by land (n = 61) or air (n = 16) to a surgical center in Germany between 2006 and 2010. All women underwent FLC on arrival at the surgical center. The postoperative survival rate for a single twin was 100.0 % (n = 16) in the flight group and 98.3 % in the land transportation group (n = 60). The postoperative survival rate for both twins was 81.3 % in the flight group (n = 13) and 75.4 % (n = 46) in the land transportation group. No differences in neonatal outcome or the rate of adverse effects were observed between the 2 groups. No flight-related pregnancy complications were recorded. It was concluded that the long-distance air travel to a specialized medical center is sufficiently safe to warrant recommendation to pregnant women with TTTS who require FLC.

Recent Advances in the Treatment of Different Fetal Diseases

Yan Chen in 2013 noted that diaphragmatic hernia complicating pregnancy rarely occurs while it is frequently misdiagnosed [36]. A pregnant woman with right upper-quadrant abdominal pain for 4 months was hospitalized because of severe unrelieved abdominal pain. Here a part of omentum and a part of transverse colon were trapped in the thorax through a patient's diaphragm. Emergency caesarean section was done and the trapped intestine, about 40 cm long, released, and a diaphragmatic hernia was repaired at the same time. The author concluded that careful examination and a timely operation are needed to treat diaphragmatic hernia complicating pregnancy.

Salim Al-Gailani in 2014 noted that health organizations and governments all over had adopted policies in order to increase intake of folic acid during the first weeks of pregnancy,

which is rich in vitamin B [37]. This folic acid supplementation has been given to reduce the Neural Tube Defects (NTDs). This article focused principally on the mandatory fortification of vitamin grain products and noting how folic acid has been transformed from a routine prenatal supplement to reduce anaemic risk to a pre-conceptional supplement to reduce birth defects. Also folic acid, which was earlier given to 'high-risk' women, is now recommended for all women of childbearing age.

Anke C. Heitkamp, 2015, reported the Splenic Artery Aneurysm (SAA) prevalence which varies from 0.01 to 10.4 %; because of asymptomatic behavior the true prevalence is uncertain [38]. These SAAs occur in younger patients with 58 % prevalence in childbearing women; 95 % diagnosed were pregnant women. The authors reported the case of a 26-year old woman thirty-one weeks pregnant, who suffered a severe abdominal pain while boarding a plane, and an urgent laparotomy had to be performed; a ruptured SAA was found and a splenectomy had also to be performed. But both mother and baby survived. The authors concluded that pregnant women with hypovolemia and acute abdomen, may be suffering from ruptured SAA. Timely multidisciplinary treatment could save the life of the mother and the child.

Discussion

Although it is now accepted that the fetus is also a patient, the specific role of surgery on the unborn as treatment of maladies is yet unclear. For fetendo surgery, it is expected that cases will increase quietly over the next few years and may decline as other approaches develop. Myelomeningocele and TTTS show promise but will be tied up in clinical trials in future. Open fetal surgery cases could double or triple if treatment is proved to be appropriate. Less invasive surgeries can become more common, because these can be mastered by practitioners who will not need specialized open fetal surgery centers. Twin-twin transfusion has the highest volume procedure and requires tertiary/quaternary expertise but if this procedure is proven effective through randomized trials, then more centers can be justified. Several models have been suggested regarding what kind of centers should be set up. The Perinatologists Surgeon Model, best exemplified at Vanderbilt, is based on treating myelomeningocele with a team including a few dedicated perinatologists, neonatologists, nurses and administrators and supportive obstetricians, neurologists and other specialists, each with specific focused duties. This model is effectively easier to implement as development of further new skills can be avoided. In another model, the Virtuoso Model, a talented practitioner uses new minimally invasive (often fetoscopic) techniques. This model requires only a well-known capable individual and can be done in a less intensive environment. What model will be cost effective etc, remains to be seen. Ultimately, trials will determine the future of fetal surgery.

Prenatal conditions will need medical management and rare conditions may need prenatal intervention to save the life of the fetus, or stop permanent organ damage. However, these are uncommon conditions, and extremely few patients might choose for fetal medical care. This implies that there is a case-load limitation.

Suggestions

The main problem is not the background of the specialist, but the to get talented individuals from disparate cultures and various medical disciplines to work together where real problems of the patients can be resolved through consultation and discussion from various angles. It is suggested that collaborative, multidisciplinary clinical and research papers can be presented to present problems and solutions at the national level.

The controversy surrounding fetal surgery today revolves around what is ideal quality and how this can be ensured, versus sufficient quantities of patients. Here the issue of advancement and option approaches again becomes pertinent. The suggestion here is to harmonize the two

approaches. This is a new exciting area where researchers and clinicians can work together for the advancement of medical practice.

Conclusion

There are a series of health issues that come under the scanner when a fetus is recognized as a patient, for instance, early recognition of a disease or defect, reducing the risk of anaemia, guidelines for prenatal nutrition, avoiding risk-aversive behaviours, overcoming genetic disabilities, encouraging newborn screening for phenylketonuria, finding out babies with CDH, diagnosing diaphragmatic hernia before caesarean section, reducing the risk of certain cancers, immediate ambulance transfer with patient information to the emergency department, multidisciplinary treatment by high skilled surgeons, to name a few.

The difficulties involved in infant and medical specialty surgery, its infrequency and the small numbers involved makes analysis of the process and consequently solely a also, very few well-designed have been performed so far. Surgery is usually an invasive procedure, and therefore many prefer different alternative approaches. But surgery on the unborn holds promise and the need of the day is to focus on how this surgical technique can be improved. It was not so long back that such surgery was unheard of; only the future will unfold its full potential.

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Reflections on Reality: Towards Scarless Healing: Déjà Vu

37

Andrew Burd

For this Chapter in this Book the story of the journey towards scarless healing should begin with the birth of Ian Donald on the 27th of December, 1910. He was born in Liskeard, an old market town located on the southern edge of Bodmin Moor in South West England. The First World War was just around the corner and within the span of one generation the world was going to be again involved in a further struggle for dominance and control. Such conflicts bring to the fore the need for science to address urgent problems of survival and there are tremendous ethical and moral issues at stake. The science of war is the science of death and destruction but also of defence and protection and it was the science of war that brought salvation to that beleaguered island in the North East of Europe; it was the use of ultrasound, 'sonar' that allowed the navel protection vessels for the vital North Atlantic convoys to detect the most formidable underwater fighters, the German U-boats and radar, a defence against the most powerful aerial fighting force the world had ever assembled at that time, the German Luftwaffe. Radar was the pioneered by a brilliant and perhaps somewhat eccentric scientist Robert Watson-Watt who was convinced that radio waves could be used to detect thunderstorms. Time and circumstances forced him to change his focus from thunderstorms to airplanes.

There were many others who were looking at these new technologies of sonar and radar and wondered about their potential medical applications and one of them was a doctor who was specialising in a field diametrically opposite from war, he was an obstetrician concerned about bringing healthy and live babies into the world. In 1954 Ian Donald, now 44 years of age, became the Regius Professor of Obstetrics and Gynaecology at the University of Glasgow. After much trial and experimentation he reported on the first diagnostic ultrasound machine in the Lancet in 1958 [94]. This new imaging device became rapidly and widely popular and a rapidly expanding field of research and development produced a stream of new machines for clinical application. Dr Joseph Woo has written the three part short history of the development of ultrasound in Obstetrics and Gynaecology and it is clear that by the 1970s the technology and its application was having a profound effect on the practice of obstetrics [104].

In 1975 another classic paper appeared in the Lancet describing the diagnosis of spina bifida at 17 weeks of gestation [92]. With the development of high definition ultrasound it was becoming increasingly possible to identify anatomic anomalies that were compatible with life in utero but were

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fatal upon delivery. A classic example would be the congenital diaphragmatic hernia, which occupies the thoracic cavity in utero [36]. This does not affect the fetus which receives oxygenated blood via the placenta. However at birth the new-born baby would not be able to inflate the lungs which would have been aplastic and have no space to expand.

Other conditions could be detected such as a urethral stricture, which would obstruct the passage of the fetal urine. Backpressure in the urinary system would prevent the renal parenchyma from developing which again would not be compatible with neonatal existence. Early identification of closed space pressure problems led to a whole new field of interventional decompressing procedures that could be performed percutaneously with ultrasound guidance. This did not address the more complex anatomical anomalies that required direct surgical intervention. This was not long in coming. The real challenge was to be able to expose the fetus through a maternal laparotomy and then hysterotomy; perform the corrective surgery and return the fetus to the uterus without initiating a spontaneous abortion.

This required extensive animal experimentation and it was in on April 26, 1981, that the first human open fetal surgery in the world was performed at University of California, San Francisco under the direction of Dr. Michael Harrison. This was a case of congenital hydronephrosis. As experience accumulated more and more complex procedures were performed but the inherent risks of interrupting a healthy and viable pregnancy, restricted the indications to life-threatening fetal anomalies. There was however a clinical observation associated with those who had undergone fetal surgery and successfully survived to term. The relevance and impact of this clinical observation cannot be underestimated in terms of the way it continues to drive a mighty sector of the medical research field. And what was the observation? The clinical observation? The wounds created surgically in the 21 week old fetus could not be found when the full term baby was delivered.

This was not an animal model. This was real and very relevant. The outcome of post-natal skin wound healing following surgery could always be identified, no matter how many years later, by the outcome of post-natal wound healing. A scar. Now scars are having a bit of a bad press in Science these days and yes indeed they are involved in a whole host of pathological processes. The problem is that scar tissue is a vital biological response to a defect in tissue integrity. You do not have the safety and the comfort, and the time that you have in the womb. The 'real' world is far more threatening and so wound closure is a priority. Now, a defect in the skin? How does that heal? Well the first point is to acknowledge that the skin is a highly complex, multifunctional organ that has a number of functional layers [41, 42]. The first and foremost of these layers is the stratum corneum. This is a layer of dead keratinocytes that provide a formidable barrier between the inner biological world and the external environment that presents far more than just biological risks. But the stratum corneum does not just happen. It is the end stage of keratinocyte differentiation, a terminal differentiation. Keratinocytes are highly organized cells and they need a specific architectural contiguity to perform their primary role, producing keratins and ultimately the stratum corneum.

So if a gap appears in the epidermis, a gap in terms of a discontinuity of functional integrity of the outer layer of the skin, the response will be to rapidly spread a new contact layer of specialised basal keratinocytes. Invading transit amplifying cells or in situ proliferation of basal cells will cover these. The horizontal cellular proliferation is halted by horizontal contact inhibition at which point the outward terminal differentiation occurs which is part of the fundamental programming of the keratinocyte which works on a regular cycle throughout life [5]. Now the end result of this rapid healing of the epidermis with restoration of the vital barrier function is indeed a structural and functional regeneration of the original epidermal architecture. Apart from slight and usually temporary disturbance in the melanocyte- keratinocyte unit organization, which may cause some pigmentary anomalies, there is no other indication that a wound has ever been present.

Postnatal wound healing of the dermis is a completely different biological process. It is absolutely reasonable to expect something very different as the dermis has very different functions from the

epidermis. It has a major biomechanical role reflected in a complex dermal architecture that provides the property of plasticity, to the skin. In terms of survival though, the barrier function of the epidermis is both key and critical so that has to be the priority. The epidermis needs a good connective tissue support and this is provided by rapid reconstitution of a supporting "dermal-like' layer which is primarily composed of the fibrillar collagens Type III and Type I [19]. These are assembled into connecting three-dimensional configurations that in the uninjured dermis support the particular properties of the dermis where the matrix, is the key and the cells are there to provide materials. When the dermis has been injured, restoring the functional integrity of the dermis is paramount and speed trumps organization. And nature did as nature does and made a compromise. I can give you rapid healing by a process we shall call repair. Repair is distinct from regeneration in that it is a healing that results in a permanent focal area of connective tissue that does not have the characteristic features of the uninjured tissue. Of course in the skin this would also include the lack of epidermal appendages, sweat glands, hair follicles, sebaceous glands that are found in Normal skin. It is this 'abnormal' region that is called scar tissue [22].

Scars come in all different shapes and sizes and have a considerable range of severity with some pathological scar types recognized, principally hypertrophic and keloid. It is important as a doctor and a scientist to acknowledge the very positive survival benefits of scar tissue formation but if you could have the same benefits without the scar? It is that goal that drives so many to try to unlock the secrets of scarless healing. It is not all humanity and compassion and if, there were a simple secret that could be elucidated, patented and commercialised the financial return would be beyond imagination. And both fame and fortune are powerful motivators for individuals working in the field of fundamental biological research.

Scarless healing has been called many things. In a review published this year it was referred to as the Holy Grail [100]. I really do not think that is a very appropriate term and at this level of science, terms, are important. The "Holy Grail" is the original chalice, or cup, that Jesus and his disciples drank from for the very last time. The night he was betrayed. The last supper. So there is a mythical, mystical link with that chalice and generations have searched for, but have failed to find it. Does it even exist?

It did. There was some receptacle used for the wine that was shared at the Last Supper. But the important point for our younger scientists who still have some neural plasticity is to appreciate that it did indeed exist. That is a matter of logic, not fact. We can't find it. Does that really matter? But there is more to it than just the semantics. It is the very limitation set upon the scientific community in the 1970s by the emerging multi-billion dollar biomedical research industry. The motivation was, too often, a romantic fairy tale of how science works. Long nights in the laboratory, repeating experiments in true reductive style, again and again, and then one day: a breakthrough. A molecule. The key to unimaginable fortune. The Corporate world has bought up so many patents based on this naively simple concept of how nature works. This was a dark time in modern science when ethics became very edgy. Retraction of papers from the world's leading scientific journals, back peddling in predictions and failures of business plan after business plan. Unit pricing of drugs, dressings devices were set at levels that assumed the funding available for purchase would reflect the need for the product. Life is not like that; no economy can support unlimited health funding.

The seduction of venture capitalists on the basis of a complete and utter fantasy really shows how divorced science had become in terms of its relevance to real life. What is this 'scarless healing' that had caught the imagination of so many scientists? In reality it is a clinical observation that has remained a constant throughout from 1985 to 2015. What about the Science? What indeed? When a science is corrupted what reality does it present? The age of the reductionism is over. Consider for the moment the logistics of preparing a baby for birth; the controls, the checks, the lists, the preparations that need to be assessed, reassessed at local, regional and systemic levels. I am of course referring to the biological processes of foetal growth and maturation which are a reflection of the complicated

nature and the biology of life. We are now in the era of 'Big Data' but we also need a more pragmatic attitude towards "truth". We still, we must, relay on facts. But the absence of a fact does not deny the presence of a possibility. Hence the Holy Grail once existed, but does it exist now?

Scarless Healing is no Holy Grail. It is a biological phenomenon which does not have a historical context out with its own intrinsic maturation. And logic and scientific evidence support the hypothesis that the scarless healing of the human fetus in the second trimester of pregnancy is an autonomous response of a more socially cohesive tissue environment where local factors, matrix factors and the cell-matrix interactions are primarily involved with the local processes of tissue generation which are perceived to be regeneration. A regeneration that is so perfect that no scar is evident within a few months.

What follows are sections of a thesis titled, "Towards Scarless Healing". This was presented for an MD degree (a higher research degree) at the University of Aberdeen in 1995. It covers work undertaken in the late 1980s and reflects the state of research and understanding about the clinical phenomenon of scarless healing in the human fetus following surgery at that time. It is a salutary lesson to look at this in the context of where we are now. In essence we are no further forward but we are beginning to realise that something as ubiquitous as scarring is going to be linked to some very basic but biologically, hugely complex, survival mechanisms. It is in this context that we are now viewing a new era of medicine which is focussed on regeneration through a biologically programmed approach using stem cell and stem cell based therapies. This is going to be a great challenge for the Health Industry as it grapples with the definitions both of pathology and of therapy based on outcomes.

Introduction and Overview

*A **wound** is a discontinuity in tissue **integrity**.*
***Healing** is the process of restoring that **integrity**.*

The specific focus of this thesis is directed at the phenomenon of scarring which, in the post-natal wound, is a constant sequel of the healing process when the integrity of the dermis is breached and dermal continuity has to be restored. The inevitability of scarring, in the post-natal wound, is now under intense investigation primarily due to the observation that foetal wounds, surgically repaired and allowed to heal in utero, can heal without scarring. In this introductory chapter the structure of skin is briefly outlined, followed by an overview of clinical skin wound healing. The major extracellular components of skin wound healing relevant to this thesis are described and finally foetal wound healing is introduced.

The experimental sections of this thesis describe work which covered a period of intensive investigation into the major differences between foetal and post natal wound healing and falls into three stages; the first two stages address specific hypotheses and the third part presents a new hypothesis for further investigation. The two hypotheses which are directly examined are:

1. The scarless healing observed in foetal animals is due to the lack of new collagen deposition in foetal wound healing [47]
2. The scarless healing observed in foetal animals is due to the hyaluronic acid rich environment which allows for more effective remodelling of the repair tissue [26].

Skin

Skin is a complex, bilaminar structure, comprising a predominantly cellular superficial layer, the epidermis, overlying a supporting layer of dense fibroelastic connective tissue, the dermis (reviewed in Odland [68]) (Fig.37.1).

The dermis is composed of collagen fibres, elastic fibres and an interfibrillar gel of glycosaminoglycans, salts and water. The principal cell of the dermis is the **Fibroblast** which synthesises and organises the major structural elements [30]. **Collagen**, which makes up nearly 80 % of the dry, fat free, weight of skin, accounts for the tensile strength of the dermal fabric [87]. **Type I** col-

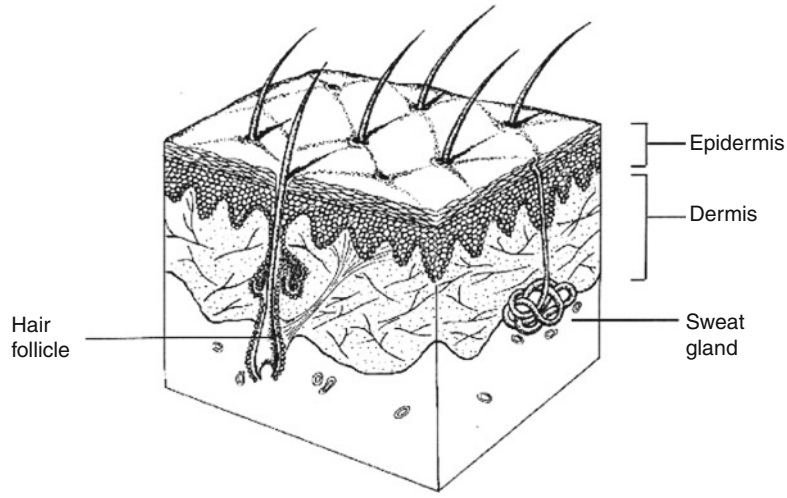

Fig. 37.1 A diagram of skin showing the bilaminar structure with the cell rich epidermis overlying the collagen rich dermis

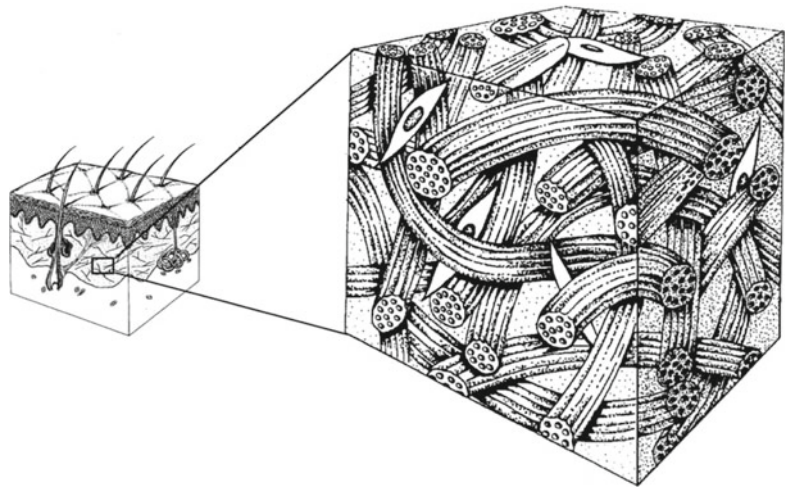

Fig. 37.2 Diagrammatic representation of the dermis showing the basket-like weave of collagen

lagen is the major collagen in the dermis with **Type III** constituting approximately 15 %. Interwoven among the bundles of collagen is a network of elastic fibres which restore the normal fibrous array following its deformation by external mechanical forces (Fig. 37.2).

The dermis is arbitrarily divided into two anatomical regions, the papillary and the reticular dermis. The thinner of these is the papillary dermis which is the outermost portion of the dermal connective tissue. It is moulded against the overlying epidermis and accordingly is thrown into surface contours of papillae and folds conforming to the basal epithelial ridges and grooves of the epidermis [6–8]. The papillary dermis contains smaller and more loosely distributed elastic and collagen fibrils than does the reticular dermis, and has a substantially greater proportion of interfibrillar gel and connective tissue cells in addition to enclosing the vast microcirculatory and lymphatic plexuses just beneath the papillary projections of the dermis. The collagen fibres of the papillary dermis are 0.3–3 mm in diameter [9]. The second region of the dermis, the reticular dermis, constitutes the greater bulk and lies beneath the papillary dermis. It is relatively acellular and avascular containing bundles of elastin and collagen fibres. The collagen fibres are considerably larger than in

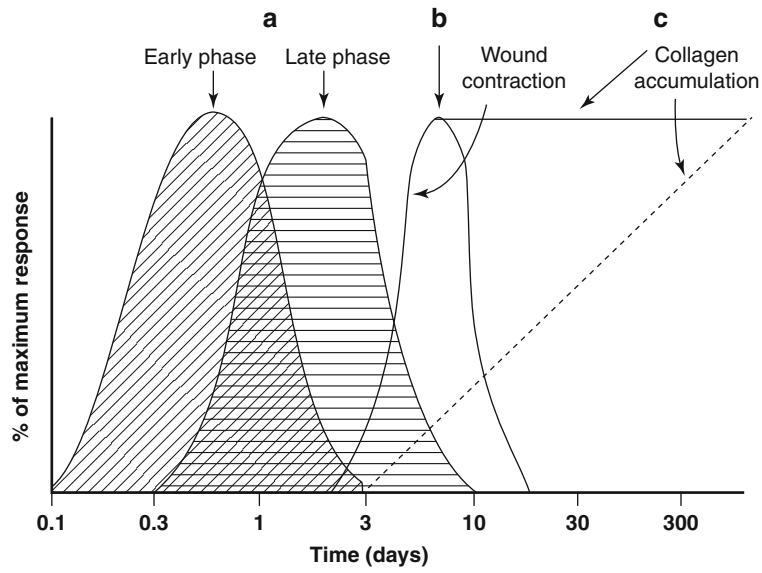

Fig. 37.3 The phase of wound repair. Three phases are arbitrarily defined: (**a**) inflammation (early and late) (**b**) granulation tissue formation and (**c**) matrix formation and remodelling [23]

the papillary dermis (10–40 mm diameter) and a proportionately smaller amount of interfibrillar gel exists.

The major macromolecular component of the non-collagenous extracellular matrix is the broad class of anionic polysaccharides [24]. These compounds, glycosaminoglycans (GAG) and proteoglycans (PG), form a diverse group of heterogeneous glycoconjugates which are formed by a protein core to which different GAG chains and N- and/or 0-linked oligosaccharides are covalently attached. GAG chains present in the ground substance of skin consists mainly of hyaluronic acid, dermatan sulphate (and smaller amounts of chondroitin-6-sulphate), heparin and heparan sulphate.

These substance play an important role in maintaining salt and water distribution; PGs control a "domain," so that a volume of water as much as 1,000 times the volume of the PG can be contained within the molecule. GAGs consist of hexosamine (either glucosamine or galactosamine), alternating with acidic sugars (glucuronic or iduronic acid). These extracellular matrix molecules are metabolised and degraded by fibroblasts and mast cells. In the dermis they exist as a viscoelastic sol-gel of tangled polymers.

Wound Healing

In the skin, there are **two basic elements to healing**;

The restoration of

(i) Cover and
(ii) Support.

New cover is achieved by re-epithelialization, new support is achieved by the deposition of a collagen rich connective tissue matrix which undergoes subsequent remodelling and results in what is recognised histomorphologically as scar tissue.

Dermal repair involves a fibroproliferative process with the re-establishment of structural integrity by the deposition of connective tissue. Classically, repair, in this context, involves three overlapping phases: (i) the inflammatory phase (ii) the phase of granulation tissue formation (iii) the phase of matrix deposition and remodelling (Fig. 37.3).

The Inflammatory Phase

This phase is characterised by a sequence of overlapping cellular events in which the breach in tissue integrity is recognised, and attempts are

made to contain and limit damaged tissue before the process of repair and reconstruction commences (reviewed in Clarke [23]). Blood vessel disruption causes:

(i) Extravasation of blood constituents and platelet aggregation
(ii) Blood coagulation
(iii) Bradykinin production
(iv) Complement-derived anaphylotoxin production (these cause the release of histamine and other granule constituents from mast cells resulting in vasodilatation) as well as having a chemotactic effect on neutrophils and monocytes.

The individual cellular elements will be described sequentially beginning with the platelets. These appear in the wound after the disruption of blood vessels results in the extravasation of blood constituents and platelet aggregation.

(i) **Platelets** are anucleate, discoid fragments approximately 2 mm in diameter derived from marrow megakaryocytes. They contain several distinct types of storage organelles containing substances which can initiate and influence the process of repair. The main function of the platelet is to initiate haemostasis by forming and maintaining a cellular plug at the site of vascular injury. Platelets adhere to the exposed sub-endothelial collagen in the vessel walls and in doing so become activated, releasing mediators that promote further aggregation and vasoconstriction. Among these mediators is platelet-derived growth factor (PDGF) which is stored in the alpha granules.
(ii) **Neutrophils** are the first leukocytes seen in the area of inflammation and injury. Despite their early and abundant presence it appears that neutrophils do not play a central or essential role in the wound healing process. This was demonstrated by the effective healing of wounds in guinea pigs rendered aneutropenic by the administration of anti-neutrophil serum [78].
(iii) **Macrophages**. Tissue macrophages are largely derived from circulating monocytes of bone marrow origin. The reason for the sequential migration of monocytes into the tissues after the neutrophil infiltration is still a matter of conjecture. Several possible mechanisms have been proposed to account for the apparent sequence: it may be that both cell types respond to the same chemotactic factors but that monocytes are slower overall, either in accumulating in the general area of the wound or specifically in migrating through the walls of the blood vessels and extracellular matrix, alternatively monocyte migration occurs later and is due to different stimuli, possibly initiated, facilitated or amplified as a result of earlier neutrophil migration.

Whatever the case it is apparent that macrophages from inflammatory sites display features of activation, tending to be larger than resident macrophages and having more secondary lysosomes, vacuoles and mitochondria. For many years it was thought that the primary function of macrophages was the removal and degradation of injured tissues prior to the onset of repair. Studies of wound healing in guinea pigs, however, have shown that the depletion of circulating monocytes and local tissue macrophages leads to a severe retardation in tissue debridement and a marked delay in fibroblast proliferation and connective tissue deposition [46, 52]. It is now apparent that wound macrophages secrete growth factors that stimulate the proliferation of fibroblasts, smooth muscle cells and endothelial cells [53]. They are therefore critical in the transition from the phase of inflammation to the phase of granulation tissue production.

Wound macrophages phagocytose and digest organisms, they scavenge tissue debris and effete PNM's in addition they release a large number of active substances:
(a) To recruit more inflammatory cells and help in tissue debridement and
(b) To initiate the formation of granulation tissue.

(iv) **Lymphocytes**. The role of lymphocytes in wound healing is unclear.

Lymphocytes are present within wounds and they do produce a wide range of lymphokines which have particular effects on macrophage and fibroblast function. Recent interest has focused on the possibility that lymphocytes and their products may be involved in abnormal wound healing seen in keloid and hypertrophic scars [61].

(v) **Eosinophils**. These cells are also seen in wounds and again their role is unclear.

Granulation Tissue Formation

Granulation tissue consists of abundant macrophages, fibroblasts, new vessels and a loose matrix of collagen, fibronectin and hyaluronic acid. Fibroblasts proliferate and migrate into the wound space. The cells alter in shape, deposit extracellular matrix materials, proteoglycans and collagen and become increasingly mobile. They form cell-cell and cell-matrix links and generate tension which results in reorganisation and contraction of the wound matrix.

Angiogenesis occurs at the same time as fibroplasia. New vessels bud from pre existing ones in a process involving a number of steps:

(a) There is enzymatic degradation of basement membrane of the parent vessel.
(b) Endothelial cells migrate towards the angiogenic stimulus (fibronectin and tumour necrosis factor (TNF).
(c) There is proliferation of endothelial cells behind the migrating cells.
(d) The endothelial cells mature and organise into capillary tubes.

The new vessels have leaky inter endothelial junctions and proteins and erythrocytes pass into the extravascular space.

Matrix Remodelling

The third and final stage of wound healing begins at the same time as granulation tissue formation. The granulation tissue is removed and the matrix altered to remove fairly rapidly most of the fibronectin present. Collagenase from fibroblasts, macrophages and neutrophils helps with the remodelling. There is a slow accumulation of large bundles of **Type I** collagen and an increase in the tensile strength in the residual scar. The numbers of active fibroblasts and new vessels decrease; many blood vessels thrombose and degenerate, the cells are resorbed and digested by macrophages.

The end result of healing is a scar composed of inactive-looking, spindle-shaped fibroblasts, dense collagen, and fragments of elastic tissue, extracellular matrix and relatively few vessels.

Extracellular Components of Repair

The foregoing account has briefly reviewed the phenomenon of wound healing. It is obviously a complex process and the mechanisms of repair in the post-natal wound are far from clear.

In this third section of this chapter I shall give an overview of the extracellular components of particular relevance to this thesis:

- Growth factors
- Collagen
- Hyaluronic acid

Growth Factors

In populations of normal cells, growth is a balance between growth stimulators and inhibitors. These can lead to a shortened cell cycle or decreased cell loss. There are an increasing number of peptide regulatory factors which have been identified in the serum and produced by cells which affect cell proliferation, metabolism and movement [67, 81]. The main growth factors are:-

1. **Epidermal growth factor** (**EGF**)

EGF is a polypeptide of 53 amino acids (MW = 6 kd) which contains three intramolecular disulphide bonds required for biological activity. The effects of EGF are mediated through cell surface receptors. EGF is mitogenic for epithelial cells and fibroblasts in culture. In vivo EGF stimulates epidermal regeneration and

collagen synthesis in a dose dependent effect [48]. The sustained release of EGF from subcutaneously implanted polyvinyl alcohol sponges was reported to result in a significant increase in granulation tissue deposition and protein accumulation [10].

2. **Platelet-derived growth factor** (**PDGF**)

PDGF is a dimer of molecular weight approximately 30,000. It consists of two peptide chains, denoted A and B, of approximately 16,000 and 14,000 molecular weight, respectively. PDGF is stored in the platelet a-granules and is released on platelet activation. It is also produced by activated macrophages, endothelium and smooth muscle cells. PDGF has both mitogenic and chemotactic activity. It is the most potent mitogen in serum for cells of mesenchymal origin, stimulating cell growth through interaction with specific high affinity receptors on the cell surface of target cells. PDGF is chemotactic for a variety of cells. The response of different cells is concentration dependent, thus maximum neutrophil chemotaxis occurs at concentration of 1–5 ng/ml whereas maximum monocyte chemotaxis occurs at concentrations of 20 ng/ml. Neutrophils are activated at higher concentrations, 20–40 ng/ml, resulting in superoxide synthesis, release of granule content and neutrophil aggregation.

It has been postulated that PDGF may play an important role in wound healing by the expression of both its mitogenic and chemotactic properties mediating the recruitment of inflammatory and connective tissue cells into the site of injury. Subsequent activation of neutrophils causes the release of lysosomal enzymes and neutral proteases which remove damaged tissues. Superoxide anions are produced which kill micro-organisms. Cellular proliferation is an essential step in repair following the inflammatory response. Fibroblasts are responsible for the synthesis and secretion of new matrix elements but also PDGF stimulates fibroblasts to secrete collagenase and this may be involved in the removal of damaged collagen and also in the remodelling process. PDGF also stimulates the production of insulin-like growth factor by fibroblasts.

3. **Fibroblast growth factor** (**FGF**)

FGF was first defined as a single factor that was mitogenic for fibroblasts. Now, there are at least five structurally related proteins having molecular weights of approximately 16,500. The most abundant is basic FGF which was initially isolated form the pituitary. Basic FGF is produced by activated macrophages; it is a fibroblast mitogen but even more mitogenic for capillary endothelial cells and can induce all the steps in angiogenesis.

4. **Transforming growth factor alpha** (**TGF**-α)

TGF-α is a 50 amino acid polypeptide that has a substantial sequence homology with EGF and binds to the same receptor protein. It has similar functions to EGF but in vivo studies suggest it is a more potent angiogenic mediator [71, 74].

5. **Transforming growth factor beta** (**TGF-β**)

TGF-β has a molecular weight of 25,000 and is a dimer of two apparently identical subunits (MW = 12,500) held together by disulphide bonds. It has a broad array of biological effects, including the ability to stimulate and inhibit the proliferation of cells in culture [69, 80]. In vivo, subcutaneous ad ministration of TGF-β induces marked collagen deposition and a single topical application of TGF-β to incisional wounds appears to promote healing. A slow release vehicle seems to be necessary for prolonged action on the wounds [65]. The application of a neutralizing antibody to TGF-β in adult rat wounds appears to eliminate scaring [76]. TGF-β is produced by platelets, endothelium, T-cells and macrophages.

6. **Interleukin one** (**IL-1**) **and tumour necrosis factor** (**TNF**).

These cytokines, both molecular weight of 17,000, produce the same spectrum of biological effects but are structurally different and do not compete for a common receptor. IL-1 is produced by nearly all cell types including lymphocytes, activated macrophages and vascular endothelium. TNF is produced by stimulated macrophages and causes production of surface adhesion molecules which result in the adhesion of neutrophils, monocytes and lymphocytes to vascular

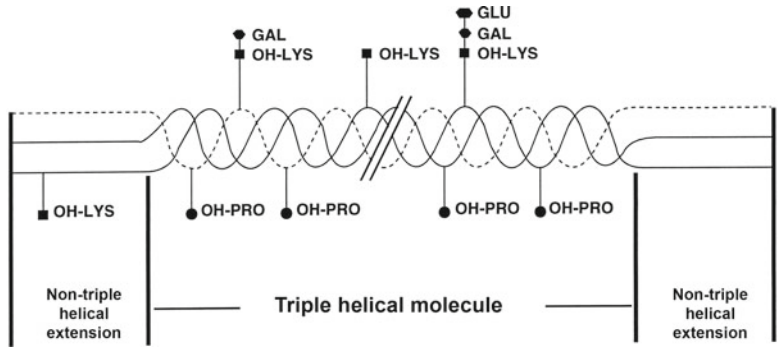

Fig. 37.4 A diagram of the major characteristics of the type I collagen molecule *OH-PRO* hydroxyprolene, *OH-LYS* hydroxylysine, *GAL* + galactose, *GLU* glucose

endothelium. It causes production of PGI2 a potent vasodilator and inhibitor of platelet aggregation. It increases synthesis of platelet activating factor. TNF produces the haemodynamic effects of septic shock and is angiogenic. It is chemotactic for fibroblasts and stimulates them to produce more collagen and collagenase. It thus has a probable role in connective tissue remodelling.

Collagen

The word collagen is derived from the Greek word Kolla, which means glue. The term collagen or glue former was originally used in the nineteenth century for the part of skin, bone, tendon and cartilage, which, when the tissues were boiled and the extracts evaporated produced glue. Collagen is a ubiquitous protein which has been the subject of extensive investigation over many years. What was once considered a relatively inert structural protein now appears to be a group of substances which have considerable in vivo metabolic potential. In order to clarify the constituent molecules regarded as collagens an inclusive definition is necessary. In general a protein can be classified as a collagen by the presence of a typical helical, collagenous domain containing peptide chains with repeating Gly-X-Y triplets, and by the presence of hydroxyproline and hydroxylysine which are relatively specific to collagens.

Collagen is the most abundant protein in the human body accounting for about 30 % of all proteins and is the major constituent of most connective tissues. In skin and tendons, collagen accounts for over 80 % of the dry weight. Since collagen constitutes the bulk of most connective tissues it makes a major contribution to their properties. The critical function of collagen is to give strength to and to maintain the structural integrity of various tissues and organs.

The Collagen Molecule

The basic unit of collagen formerly known as tropocollogen is now known as the collagen molecule. It is shaped like a pencil with a diameter of 15 Å and a length of 3,000 Å. The collagen molecule consists of three polypeptide chains that are coiled into a unique type of helical structure (Fig. 37.4). The most abundant body collagen, Type I, has a collagen molecule with two chains of one type, designated alpha 1, and one of another type, designated alpha 2. Both chains are almost identical, but their amino acid compositions differ enough for the chains to be separable by ion exchange chromatography.

The alpha chains each contain about 1,000 amino acids and therefore have a molecular weight of about 95,000 and each chain is characterized by the presence of the typical GLY-X-Y repeating sequence throughout 90 % or more of its length. Proline and hydroxyproline follow each other relatively frequently and the Gly-Pro-Hyp sequence makes up about 10 % of the molecule.

Glycine constitutes 33 % of the amino acid residues in collagen. Twenty-two percent is made up of the imino acids, Pro and Hyp. Other unique features of the amino acid composition of collagen are the presence of hydroxylysine (Hyl) a low content of tyrosine and the absence of tryptophan. Cysteine is also absent which eliminates

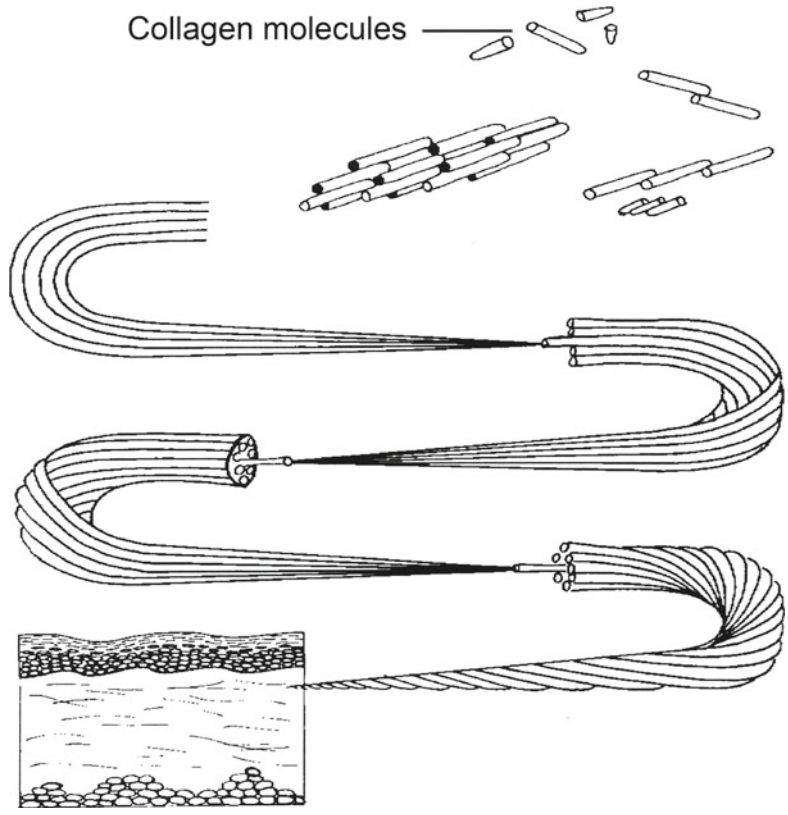

Fig. 37.5 A representation of collagen molecule aggregation into fibrillar bundles and eventual fibre formation. These interwoven bundles give structure and strength to the dermis

the possibility of disulphide cross-links in the molecule. Both Hyp and Hyl have been considered specific to collagen, but they do occur in very small quantities in other animal proteins (elastin, the Clq component of complement, acetyl cholinesterase, glycoproteins obtained from lung lavage and a collagen-like protein absorbed to human blood cells).

The biosynthesis of collagen is a complex process. It is apparent that each collagen is coded for by a different and specific gene thus chromosome number 17 contains the coding information for the alpha-l and alpha-2 chains of Type I collagen. After the gene is transcribed, it is spliced to yield a functional mRNA which contains about 3,000 base pairs. This is the template for the formation of the amino-acid sequence. Post translational changes occur to the amino-acids in this sequence which is going to be unique for an individual collagen alpha chain. Hydroxylation of proline and lysine is particularly important. The responsible enzymes are prolyl and lysyl hydroxylases. Their substrate is oxygen and co factors are ascorbic acid, ferric iron and alpha-ketoglutarate. Without ascorbate, the collagen molecule can be synthesised up to but not beyond the point of praline and lysine hydroxylation. The degree of hydroxylation varies from tissue to tissue depending on substrate availability, rate of synthesis and turnover time.

Glycosylation of hydroxylysine then occurs and is catalysed by galactosyl transferase and the addition of glucose by glucosyltransferase. Once synthesis of the individual pro-a chains has occurred, formation of the triple helical structure ensues. The procollagen is then passed through the cisternae of the rough endoplasmic reticulum and through a transitional endoplasm to the Golgi where it is packaged into secretory vesicles prior to extrusion by exocytosis.

Once the molecule has left the cell the terminal, or registration peptides are cleaved and it becomes involved in a process of organisation with the formation of intra and inter molecular cross-linkage (Fig. 37.5).

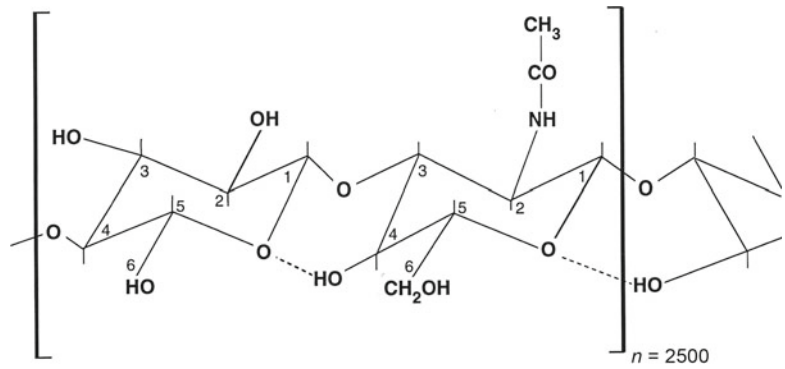

Fig. 37.6 The repeating disaccharides of hyaluronic acid

Crosslinking

Crosslinking stabilises the collagen fibres giving them an adequate degree of tensile strength and visco-elasticity to perform their structural role. The process involves an aldehyde-mediated mechanism with the conversion of specific lysine and hydroxylysine residues in collagen to peptide-bound aldehydes. Enzymes responsible in this step are lysyl hydroxylase and lysyl oxidase which converts the hydroxyl group to an aldol and initiates the lysine-lysine linkage.

Hyaluronic Acid

Hyaluronic acid (HA) was first isolated by Meyer and Palmer in 1934 [63], and has been shown to be a ubiquitous constituent of connective tissues. The chemical structure was established by enzymatic [64] and chemical [43] studies carried out on preparations obtained from human umbilical cord and consist of alternating 4-0-linked b-D glucopyranosyluronic and 3-0-linked 2-acetamido-2-deoxy-b-D-glucopyranosyl residues (Fig.37.6).

Because of its composition of hexosamine and uronic acid, and also of its biosynthesis, it has been classified among the glycosaminoglycans such as dermatan sulphate, chondroitin sulphate, heparin sulphate, heparin, etc., although it is the only polymer of this class of compounds in which a linkage to protein backbone (to form a proteoglycan) has not been demonstrated [24]. The history of the structure determination of glycosaminoglycans, where new sugar components or new anomeric configurations have been found repeatedly, suggests that variations in the structure of HA are possible.

Recently N-deacetylated HA has been reported in old human skin [60]. Although a linear polysaccharide, its macromolecular structure is that of an expanded coil. This is susceptible to enzymatic degradation, resulting in the release of low-molecular weight oligosaccharides. HA and its degradation products have been associated with a number of cell regulatory actions. Considerable attention has been directed towards the role of HA in growth and development [84], and it would appear that the structural configuration of hyaluronic acid facilitates the movement of cells within the extracellular matrix [28].

Now increasing attention is being directed towards the role of HA in wound healing [4]. In the early stages of repair, when HA levels are high, cell migration plays a prominent role in restoring cellular continuity [1]. Later, when HA concentrations fall and sulphated GAG concentrations are high, cell differentiation, collagen production, and tissue organisation occur [82, 83]. The high molecular weight HA seems to inhibit the formation of capillaries [29] while angiogenesis is induced by degradation products of HA [88]. Weigel et al. [86] have proposed a model for the role of HA in wound healing in which an HA-fibrin matrix forms which attracts inflammatory cells into the wound. This matrix is in turn modified by the cells entering the wound as they secrete hyaluronidase and plasminogen activator into the extracellular space to degrade hyaluronan and fibrin. The degradation products from both HA and fibrin are then seen as important regulatory molecules for controlling cellular functions involved in the inflammatory response and inducing new blood vessel formation in the healing wounds.

There is now evidence to suggest that postnatal wound healing can be modulated, resulting in a

decrease in scarring. Hellstrom [40] reported enhanced wound healing in tympanic membrane perforations in a rat model following the topical application of tissue-extracted hyaluronic acid (HA). Membranes treated with HA appeared otomicroscopically normal (translucent) 3 months later in contrast to untreated membranes which showed extensive opacification. In a follow up study it was reported that this effect was independent of molecular size or rheological properties of the HA but related to the concentration of the preparation [50]. Abatangelo et al. [1] reported that HA facilitated wound healing in diabetic rats. Its mode of action appeared to be the promotion of epithelial migration and differentiation. Radaelli et al. [70] using a rat model reported that HA treated wounds developed a greater early wound breaking strength compared to untreated controls. The reason for enhanced wound strength was due to an early accumulation of oriented collagen fibres.

Foetal Wound Healing

Human foetal surgery has been successfully performed in a small number of highly selected cases for foetal urinary tract obstruction, diaphragmatic hernia and sacrococcygeal teratoma, which threaten foetal viability [37, 38, 49]. This experimental therapy involves maternal laparotomy and hysterotomy, delivering the foetus and per forming the necessary foetal surgery, after which the foetus is returned to the uterus and the pregnancy allowed to continue. At subsequent delivery, it was observed that there was a lack of scarring and contracture associated with the surgery (M. R. Harrison., personal communication). The absence of scarring makes the prospect of reconstructive human foetal surgery very attractive [20], but the inherent risks of interrupting a healthy and viable pregnancy, at present, restricts human foetal surgery to life-threatening foetal anomalies [21, 25, 32–34, 39]. Perhaps more relevant, at this stage, is investigating the biology of foetal wound healing to discover the mechanisms of scarless healing. The aim of such studies is to discover insights into the modulation of postnatal wound healing and, thus, decrease scarring, a matter of relevance to all surgeons [55].

It must be said, at the outset, that the fundamental mechanisms of foetal wound repair remain a mystery. To date, they have been described in terms which are appropriate to postnatal repair and, yet, there do seem to be a number of major differences between the foetal and post-natal repair. From the point of view of the immunohistochemical and biochemical analysis, there are well described differences between foetal and post-natal repair both at cellular and extra cellular levels [3, 44, 45, 47, 56, 57, 59, 72].

In the foetal wound healing model there is a minimal inflammatory response with little deposition of granulation tissue or fibrosis seen [18]. The role of collagen was a subject of major controversy in the mid-1980s and is examined in some detail in this thesis. The glycosaminoglycans content of the healing wound in the foetus has been the subject of intensive investigation and it has been noted that hyaluronic acid is deposited during early stages of foetal repair and that fibronectin and tenascin deposition are early features of foetal wounds [89–91].

The investigation of foetal wound healing is moving on from phenomenological studies to more mechanistic investigations and the focus of interest now is directed more towards what makes the foetal wound heal. Studies that look at the regenerative as well as the developmental and oncogenic processes have uncovered some intriguing aspects of cellular control that might have significance in foetal wound repair. Homeobox genes are a class of genes which code for peptide regulatory factors that bind with varying degrees of specificity to DNA and promote the transcription of structural and/or metabolic genes. The idea, basically, is that in foetal wound repair there is reformation of injured tissue to create the architecture of the pre-injury tissue. The central role of DNA in the process of co-ordinated production of organised tissue is a matter of some debate. Some investigators regard the DNA as the ultimate controlling factor in tissue organisation. The predominance of the cell, however, is not universally accepted and other investigators feel that the extra-cellular matrix has a profound influence upon the ultimate tissue architecture which results from the co-ordinated repair of a tissue defect.

In essence, then, the process of foetal wound repair is more usefully recognised as a process of regeneration rather than the healing and the regulatory mechanisms for regeneration are, in all probability, very different from those for repair.

As mentioned in the introductory paragraphs to this chapter, this thesis is a historical record that describes the experimental work which had a major influence in directing the focus of research in foetal wound healing.

The Essential Difference Between Foetal and Post-Natal Wound Repair Appears to Be Not What Is Deposited but What Happens to It

In the foetus reorganisation of repair tissue results in the restoration of the original tissue architecture.

In the post-natal wound, reorganisation of the repair tissue does not result in the restoration of the tissue architecture.

In the former **regeneration** has occurred.

In the latter there is the formation of **scar tissue**.

Foetal Rabbit Studies

Introduction

It is perhaps surprising that the presence of collagen in the foetal wound healing process has been questioned. Krummel et al. [47] reported no detectable hydroxyproline (a highly specific marker for collagen) using a highly sensitive liquid chromatography analytic technique [54], in silastic/poly vinyl alcohol (PVA) tubes implanted in foetal rabbits. Adzick et al. [3] had looked for collagen deposition in another wound healing model in foetal rabbits and had reported elevated levels of hydroxyproline. Adzick had used implanted polytetrafluoroethylene tubing [31]. The histological events in both wound healing models are reported to be similar to those found in incisional wounds. Adzick had not been able to characterise the collagen although it was apparent that the reported yields of collagen in the Gore-Tex tube model were well within the limits of detection of a micro analytical typing technique which I had developed under the guidance of H. Paul Ehrlich, Director of the Wound Healing Laboratory at the Shriners Burns Institute, Boston. This technique involved the typing of collagen by the chromatographic separation of peptides derived from cyanogen bromide digests of tissue samples. Adzick's work was repeated in collaboration with Dr John Siebert MD, a resident in Plastic Surgery at the Institute of Plastic and Reconstructive Surgery at New York University, New York. The foetal animal surgery was performed in New York and the harvested material was delivered to Boston for histological and biochemical analyses.

Amino acid analysis (residues per 1000) (40 pmole sensitivity)

	Foetal		Newborn	
	Day 4	Day 7	Day 4	Day 7
HPro	**1**	**4**	**4**	**33**
Asp	89	55	94	77
Thr	52	55	51	41
Ser	54	55	51	46
Glu	116	126	125	109
Pro	57	60	62	78
Gly	67	78	72	160
Ala	99	90	92	92
Val	74	65	69	55
Cys	27	0	32	13
Met	3	12	2	3
Ile	34	37	35	29
Leu	100	90	71	69
Tyr	29	27	30	20
Phe	37	35	34	26
His	28	47	37	28
HLys	**O**	**1**	**4**	**4**
Lys	96	95	91	71
Arg	39	35	45	46
Collagen (ng/tube)	41.5	43.5	198	291
	$n = 22$	$n = 8$	$n = 24$	$n = 24$

This table reflected the amino acid analysis of the Gore-Tex tube model.

Discussion

The findings presented here, again using a rabbit model, do not support Adzick's work which reported abundant quantities of collagen in Gore-Tex implants. At the same time, this study does not support Krummel's concept that collagen is not involved in the healing process in the foetus. The Gore-Tex tube implant model has been criticized because of a tendency to be encapsulated leading to poor ingrowth of new tissue [27]; however, this was not our experience. One observation is that when using the Gore-Tex tubing, care must be taken to ensure that the tissue surrounding the tubing is not analysed with the content of the tubing. Incomplete removal of the connective tissue capsule from the outside of the tube before analysis, would contribute to elevated hydroxyproline levels attributed to the tube contents. The silastic/PVA model overcomes this problem by enclosing a PVA sponge within a perforated silastic tube. The tube forms an inert zone between the sponge and the surrounding tissue. Only new tissue will grow into the sponge which can be isolated intact from the surrounding tubing. One possible explanation for the reported absence of hydroxyproline, despite a very sensitive analytical technique, relates to sample size. Preparative steps, in the technique used to process the PVA sponges, result in dilution of the sample. Under such circumstances the detection limits may have been reached due to dilution. In our study, amino acid analysis failed to detect hydroxylysine in the foetal Day 4 samples although hydroxyproline was detected. In the Foetal Day 7 sample both were detected. Hydroxyproline is not unique to collagen. It occurs in other proteins [2]. The combination of hydroxyproline and hydroxylysine, however, supports the presence of collagen. In addition there is the morphologic appearance of the striated fibre on electron microscopy. Native collagen does have a banded appearance with a periodicity of 67 nm. The finding of aperiodicity of 54 nm does not exclude the possibility that this is fibrillar collagen. Fixation artefacts can affect the observed periodicity. The presence of collagen deposition and the absence of scarring following foetal repair would suggest that the process of reorganization of the tissue at the repair site is far more efficient than in the postnatal healing process. Another possibility is that healing in the foetus occurs by a process of regeneration as suggested by Rowsel [73]. In terms of reconstitution of a dermal matrix, repair with a high degree of reorganization or regeneration, would produce the same result. However, skin is far more than a layer of epithelium over a dermal matrix. Our histological findings support the concept of a high degree of matrix reorganization, but the decreased numbers of appendageal elements suggest that true regeneration has not occurred.

While the evidence suggesting the presence of collagen within the wound healing model in foetal rabbits remains, it was obvious that very small amounts were present which precluded further characterization. It was thus decided not to pursue further biochemical studies using this animal model but explore the possibility of gaining further information from a larger animal model, the foetal sheep, which is described in the next section.

Foetal Sheep Studies

Introduction

Adzick and Krummel had come to diametrically opposite conclusions from their studies of collagen deposition in similar foetal rabbit models, they had however used two different wound implant models. We had been unable to reproduce Adzick's findings using the similar animal and wound healing models and yet had not been able to support Krummel's conclusions. At this time there were more reports coming from other investigators which tended to increase rather than resolve the controversy regarding the presence and role of collagen in scarless wound healing in the foetus. These studies all involved small animal models [3, 35, 47, 62, 66, 73, 77].

One possibility was that the conflicting results were associated with the small size of the animal models used and in order to finally resolve the controversy it was decided to use a

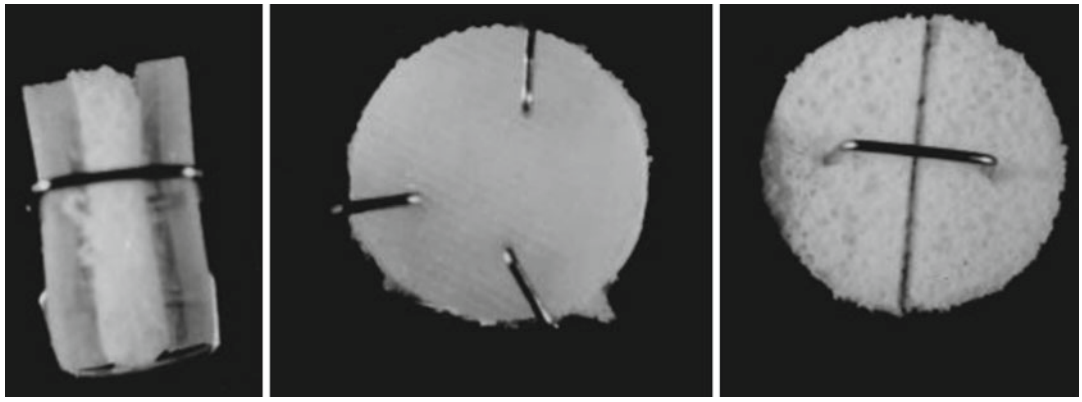

Fig. 37.7 The wound healing model showing the sandwiched sponges, from the side (*left*), and above (*centre*) and the bisected sponges (*right*)

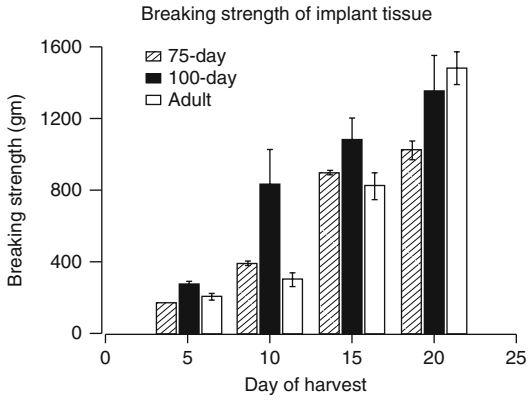

Fig. 37.8 Breaking strength of the wound tissue in the sponge implants (*error bars* ± SEM). Six sponges from each time point were used and the results are the mean of six values

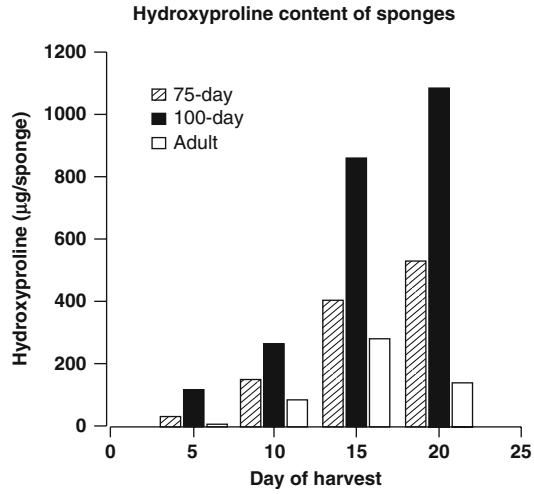

Fig. 37.9 Hydroxyproline levels in implants from a 75-day foetus, a 100-day foetus and an adult. For this assay, the sponge halves from the breaking strength assay were pooled for each time point and stage of gestation

well-characterized large animal, the sheep [36], to investigate the deposition of collagen in a model of wound healing (Figs. 37.7, 37.8, 37.9, and 37.10). This study was again a collaborative venture with the animal surgery being performed in San Francisco by Michael Longaker, MD. at the Fetal Treatment Program, University of California, and the histological and biochemical analysis of the harvested tissues being performed in Boston.

Wound Healing Model

Two configurations of polyvinyl alcohol sponge (PVA) discs (12 mm×3 rnrn) were implanted subcutaneously, sponges were either cut in half, and the two halves re-joined by a staple, or an intact sponge was sandwiched between two equal-sized silicone rubber discs. The "sandwiched" sponge allows new tissue deposition to occur only from the periphery of the sponge. Histological studies of PVA sponges implanted in adults have demonstrated that the cellular and matrix sequence of events in the sponges reflects the events of incisional wound healing in postnatal skin [75].

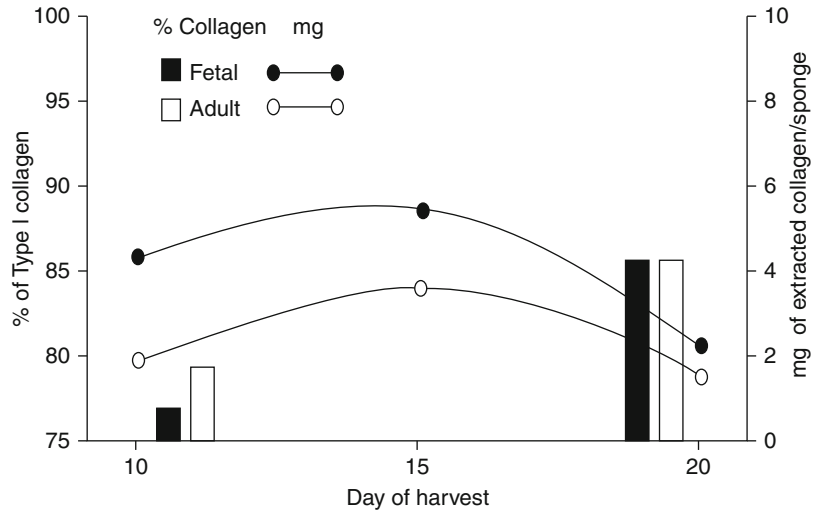

Fig. 37.10 A comparison between the content and composition of the collagen in adult and foetal sponges using conventional extraction techniques. The x-axis on the left shows the % of Type l collagen. This is a more rigid form of collagen and is associated with more mature or stable forms of extracellular matrices. The % is demonstrated in the histogram. The *curves* demonstrate the amount of collagen with the value expressed in mg of extracted collagen/sponge in the right side x-axis

Discussion

Evidence for the deposition of collagen in this wound healing model in foetal sheep is presented both histologically and biochemically [14]. Other investigators have looked for collagen deposition in foetal wounds in small animal models. Rowsell could find no evidence of new collagen deposition in foetal rat wounds using trichrome staining [73]. This histochemical stain lacks the sensitivity of currently available immunohistochemical stains, which have now demonstrated the presence of Types I, III, and VI collagen in incisional wounds in foetal mouse and sheep. Trichrome staining in our study was useful because of the abundant deposition of collagen in the sponges.

Merkel and colleagues reported the presence of collagen from pooled tissues from excisional wounds in foetal rats [62]. Their study was not supported with histological evidence that the biopsied material was definitely wound repair tissue rather than fascia underlying the wound. Previous studies with foetal rabbits show that excisional wounds do not close and that granulation tissue is absent [79]. These small animal studies were all performed late in gestation. When collagen deposition was reported, the yield was too low in individual animals to permit qualitative analysis.

The use of a large animal model, such as the sheep, facilitates surgical manipulations and allows more extensive analysis of wound tissue. The sheep has a 145-day period of gestation compared to 31–32 days for the rabbit. Foetal surgery can be performed, with a low abortion rate, as early as 55 days in the sheep (early second trimester), compared to 22 days (early third trimester) in the rabbit, thus giving a much longer time to follow the wound healing process.

It should be noted that these studies have been carried out on animals which represent a range of embryonic development. This is reflected in the varying rates of maturation of different body systems in utero. This makes inter species comparisons difficult as well as making extrapolations from animal studies to the potential behaviour of human tissues uncertain. That being stated it should be noted, as mentioned in the introduction, that scarless healing has been observed in human foetuses.

Since collagen is the major component of scar tissue in post-natal animals, how does scarless healing proceed in foetal animals, which also lay

down a collagen-rich repair matrix? Perhaps the first point to emphasise is that in healing incisional wounds in foetal sheep the initial repair tissue deposited, is disorganized [58]. For up to a week, histologically, scar tissue is present but by 14 days this is no longer apparent. So there are similarities in the fundamental processes of healing in adult and foetal animals; a defect is quickly filled in with connective tissue components which are subsequently rearranged to effect a more permanent repair. The nature of the repair in the foetus will be determined by the composition of the materials used and the manner in which they are rearranged. The findings in this section demonstrate some qualitative and quantitative differences in the collagens deposited in foetal and adult wounds.

This work disproves the **first hypothesis** that the scarless healing observed in foetal animals is due to the lack of new collagen deposition [47]. This thesis, however, continues with the exploration of the phenomenon of scarless healing and the next chapter considers the **second hypothesis** that the scarless healing observed in the foetal animals is due to the hyaluronic acid rich environment which allows for more effective remodelling of the repair tissue [26].

The Role of Hyaluronic Acid in Wound Healing

Introduction

The second hypothesis to be explored was proposed by DePalma et al. [26] from the Medical college of Virginia. This group, using a foetal rabbit model, had attempted a characterisation and quantification of the wound matrix in the foetal rabbit and had reported high levels of hyaluronic acid. They proposed that the scarless repair in the foetus was due to a regenerative process facilitated by a wound matrix rich in hyaluronic acid (HA). We had confirmed the elevated levels of HA in the Gore-Tex tube implants but were also intrigued by reports of the modulating effect on post-natal healing achieved by the topical application of tissue extracted HA resulting in reduced scarring. Hellstrom reported (1987) reduced scarring in tympanic membrane perforations in an adult rat model. Topical HA treated membranes appeared otomicroscopically normal (translucent) 3 months after treatment in contrast to untreated membranes, which showed extensive opacification. Laurent et al. [50] reported that closure time for the perforations was related to the concentration of HA but not to its molecular weight or viscosity. Also using a rat model, Radaelli et al. [70] reported that HA-treated wounds developed a greater early wound breaking strength compared to untreated controls due to an accelerated accumulation of oriented collagen fibres. These experimental studies were performed using HA extracted from rooster comb.

HA is present in all tissue in varying concentrations, with the highest concentration occurring in soft connective tissue [51]. Although a linear polysaccharide, its macromolecular structure is that of an expanded coil, which makes it susceptible to enzymatic degradation, resulting in the release of low molecular weight oligosaccharides. HA and its degradation products have been associated with a number of cell regulatory actions. In the past considerable attention has been directed towards determining the role HA plays in growth and development [84]. Currently attention is beginning to focus on the function HA serves in wound healing. In the early stages of repair, when HA levels are high, cell migration plays a prominent role in restoring cellular continuity. Later, when HA concentrations fall and sulphated GAG concentrations increase, cell differentiation, collagen production, and tissue organization occur [82, 83]. A high molecular weight HA seems to inhibit the formation of capillaries [29], while angiogenesis is induced by degradation products of HA [88]. A theoretical model has been proposed by Weigel et al. [86] for the role of HA in wound healing; an HA-fibrin matrix forms that attracts inflammatory cells into the wound, and this matrix is then broken down by cells entering the wound. These cells secrete hyaluronidase (Hyase) and plasminogen activator into the extracellular space to degrade HA and fibrin. The degradation products from

both HA and fibrin are viewed as regulatory molecules for modulating the cellular functions involved in the inflammatory response and inducing new blood vessel formation in the healing wound.

The question remains, however, concerning the mechanism for the proposed effect of HA in post-natal wound healing and scarring? The possibilities are:

1. Macrophage-effected postnatal repair process is suppressed,
2. An intrinsic tissue repair process is augmented, or,
3. A combination of the two effects occurs.

Whatever the case, a mechanism must be proposed where HA has a definite role in harnessing and manipulating the natural reparative capacity of tissue fibroblasts. This raises the question whether HA alone, has the capacity to control events of such complexity as wound healing and scar maturation. Preliminary studies have identified a heterogeneous group of HA-protein complexes in normal skin and post-burn scar [11]. The association of highly purified HA and protein is well established [85]. The protein composition of these complexes suggest that it is not random contamination; collagen comprises <5 % of the protein in the complexes, yet is the most abundant tissue protein. A functional association is suggested by the resistance of the collagen in the complexes to collagenase digestion.

Discussion

This study demonstrates that HA produced in the clinical laboratory from human tissue extracts or commercially from human and animal extracts varies both in its biochemical composition and also in its biological activity. A major contributing factor dictating this variation would appear to be the proteins associated with the HA. When an HA-protein complex extracted from human scar tissue was added to an in vitro, foetal sheep, wound healing model, there was gross evidence of a resulting dermal repair [16]. The relevance of this experiment concerns the putative nature of the foetal tissue wound healing process. As it demonstrates more autonomy, what directs the fibroblasts in the repair process? It would appear that it is not a self-directed process in that the fibroblasts are present and viable in the control wounds but healing of the dermal matrix does not occur. Similarly the possibility of epidermal control of the dermal healing process can be excluded as the epidermis is viable, proliferates and restores continuity but again no dermal matrix repair occurs. DePalma and colleagues have suggested that the HA enriched environment of the foetal wounds creates a permissive environment for dermal repair [26], however, the application of HA alone does not result in dermal repair. It is only when tissue extracted HA containing associated proteins, is added that healing occurs. This suggests that even in the foetal wounds, extrinsic factors are important in the initiating repair and it is possible that macrophages play a role in this process in vivo.

General Discussion

The thesis began by exploring the hypothesis that foetal and postnatal wound healing were fundamentally different processes due to a lack of new collagen deposition in foetal wounds. When we consider the precept that a wound is a 'discontinuity in tissue integrity' it does appear strange that such a hypothesis should be advanced as the major structural contribution to tissue integrity is indeed collagen. The hypothesis, however, was proposed by several investigators who looked for histological and biochemical evidence of collagen deposition in several wound healing models and found none. The observation that stimulated this investigation was that incisional wounds in foetal skin appeared to heal in utero without any evidence of scarring.

The experimental aspect of the thesis began by exploring this hypothesis using a small animal model, the rabbit. The conclusion of the work described in Chap. 2 is that collagen deposition does occur in this animal model but the amounts were very small. It was only when the results of the large

animal study described in Chap. 3 were presented that the controversy was finally put to rest [11].

A new hypothesis was thus needed to explain the difference in the wound healing processes in adult and foetal animals which led to the scarring in the post-natal situation and the scarless healing in the foetal model. At this stage in the evolution of the thinking about the differences in foetal and post-natal wound healing another interesting observation was being made and verified in a number of animal studies, namely that the healing of post-natal wounds could be modulated by the application of tissue extracted HA, resulting in a decreased amount of scar tissue. The question thus arose, what is the relationship between HA and the organization of the repair tissue. Was it purely physical, promoting an environment in which the new collagen could be more easily manipulated and organized? Was it chemical? Or was it not the HA at all or at least not entirely the HA? The second hypothesis that is examined in this thesis is that it is the hyaluronic acid rich wound matrix in the foetus which promotes the scarless repair.

The experimental work presented in Chap. 4 does not support this hypothesis. A number of experiments are described looking at the effect of tissue extracted HA on epidermal cells, fibroblasts and in vitro wounds. The conclusion is that it is the proteins associated with HA which appear to have biological effects which persist even when the HA is enzymatically degraded. These effects are, however, lost when the complex is heated, presumably due to denaturing the protein.

This then is the extent of the experimental work of the thesis and several points can be made regarding the possible manipulation of the wound healing process:

1. Foetal and post-natal wound healing process are fundamentally similar.
2. The **MAJOR difference** is in the organization of the collagen which is deposited.
3. Animal studies suggest HA stimulates reduced scarring in post-natal animals.
4. The in vitro studies described in this thesis suggest that the reduced scarring may be a function of the activity of the associated proteins.
5. If scarless healing is going to be achieved in the post-natal wound then two stages must be considered: the work presented in this thesis strongly supports the idea that in the foreseeable future it will be possible to suppress the adult type response to injury by targeting a specific factor(s) and in a similar fashion it will be possible to augment/enhance the foetal response which results in neogeneration rather than repair.

This leads to a **new hypothesis**:

Scarless healing in skin wounds in post-natal animals will be achieved by the application of appropriate peptide regulatory factors which will suppress the response to repair and allow a foetal -like ability to generate new tissue.

How can this hypothesis be explored?

Firstly a working hypothesis has to be proposed: and this is that scar maturation, as a function of matrix remodelling, is determined by cell matrix interactions which are, in turn, determined by peptide regulatory factors. The questions that need to be addressed are:-

1. What peptide regulatory factors are involved in matrix remodelling?
2. How do these factors produce their effects?
3. How can these factors be extrinsically modulated to reduce scarring.

To address the first question it would be possible to continue to look at the proteins associated with highly purified, tissue extracted, hyaluronan (HA) from post-burn scar tissue of varying stages of maturity. I have proposed that this HA-protein association represents a select ‘window’ into the biological activity of the remodelling matrix [11, 15, 17].

The second question could be addressed in two ways:

Firstly, the tissue extracted proteins could be assessed individually and in combination using a variety of in vitro and in vivo models including monolayer cell proliferation studies, the fibroblast populated collagen lattice

(splinted and unsplinted), and incisional wound repair using tissue explants [13]. Small and large animals could be used in in vivo studies, incorporating incisional wound and PVA sponge implant models [12, 75]. Wound healing could be assessed biomechanically and histologically using light and electron microscopy and immunohistochemistry.

Secondly, individual and combinations of known, and well characterised, peptide regulatory factors could be assessed using the same models. The third question would require in vitro and in vivo animal models but eventually clinical studies would be required.

This chapter has described part of the journey towards the ultimate clinical goal of scarless healing. The prospect of modulating the healing of surgical incisional wounds is very encouraging. The goal extends far beyond this however, to the healing of those with more extensive injury particularly the scarring which results from burns. When this affects the face, the resulting deformity can lead to the isolation of the patient from society. When the scarring affects the hands, the disability from functional loss can lead to the loss of work and economic deprivation. The aim then, of this work is to gain further insights into the fundamental biology of wound healing and scar maturation.

To reduce or eliminate scarring is to give new life, new hope, to those countless victims of trauma whose future, at present, is characterised by deformity and disability.

Summary and Conclusions to "Towards Scarless Healing: Déjà vu"

Science has undergone some major changes in the last 30 years and now must come to terms with the major limitations in the classical reductionist view. Biological events are highly sophisticated and complex processes where neither cell nor matrix reign supreme. The molecular biologists must recognize the power and the influence of the biology of molecules which have a profound effect on the outcome of such broad concepts as tissue regeneration and tissue repair. It is also essential to understand the key role of clinical observation as an ultimate arbiter in determining life and death (literally). Whilst clinical observations seem to stand the test of time, scientific observations are more transitory in nature relating to both the prevailing understanding of biological processes and the ability to measure them.

The extrapolation of research from small animal models to further the understanding of potential therapeutic manipulations in human post-natal wound healing continues to cause concern. A point to note is that when the fetal skin is injured in the third trimester of pregnancy then scarring does result. That scarring begins to become apparent when polymorphonuclear leucocyte cells appear in the fetal circulation might suggest that the fetus is getting ready for the changing priorities of tissue repair once the ex-utero status has been achieved. To attribute the presence or absence of microbiota to acceleration or delay in wound healing and the outcome in terms of scarring or no scarring might be supported by studies of genetically manipulated mice [93], however does it fit in with the reported clinical observations? The lack of clinical understanding becomes of greater concern when reports of research from globally prestigious centres claim to hold a "contrarian view" that inflammation might even be a positive benefit to regenerative healing (also known as scarless healing). To give apparent clinical relevance to very sophisticated molecular biological studies again on mutant mice by suggesting the observations should promote some future clinical studies on the effects of NSAIDs on wound healing is bordering on the surreal [97]. This also illustrates the gulf of misunderstanding between the "Ivory Towers" and the wards and clinics where hundreds of thousands of patients present each day seeking treatment for the care of wounds both acute and chronic. It is perhaps also somewhat disappointing to find that science and scientists have fixations on "magic molecules" that singularly and uniquely will unravel the mysteries of the universe. Do such molecules exist? TGF-β and its various isoforms continue to excite research scientists particularly

when they can be put in the context of a fibrosis-associated canonical pathway [101]. But how specific, reliable and relevant are such pathways when the reality of cellular context, the multiplicity of signalling events and potential multiple outcomes have rendered the concept of linear pathways to the garbage bin. The new era of science is going to have to come to terms with the reality of complex programs of response that are going to need very different methods of analysis than are currently practised. It is important to think "out of the box" but in so doing it is important to maintain the clinical perspective. Looking at the healing of the oral mucosa is a good example. Clinical observation confirms that post-natal scarless healing occurs both with a regularity and predictability following injury to the oral mucosa. But the human oral mucosa is critically different from the skin in not having to provide such a vital and complex barrier function. Again it is disappointing to read published research which reveals a lack of basic knowledge of the clinically observed phenomena; to state that "foetal wounds heal fast without scar formation" is just not true [95, 96].

Recent reviews on scarless healing make little or no mention of the collagen story revealed in this thesis nor are the needs for different forms of wound healing given appropriate consideration. The conclusions are little different from 20 years ago, "The precise mechanism of fetal regulation remains unclear with a number of differences identified between the fetal and adult wound healing" [99]. These authors did acknowledge that the introduction of stem cells is going to change the game. Stem cells can provide a multiplicity of factors which involve post-translational modifications and matrix organization. This is a very important point to keep in focus as ultimately the scar is a matrix and not a cellular phenomenon [102]. Another review which again overlooks the original collagen question does correctly identify the control of the extra cellular collagen matrix as a key challenge. They propose educating adult fibroblasts to behave like fetal fibroblasts to reduce scarring through cell therapy [105]. This seems to partially reflect the hypothesis proposed over 20 years ago that the assembly of the dermal architecture is more likely to be achieved when the biological imperative of rapid and strong repair is overruled. The interaction of fibroblasts and keratinocytes remain a target for exploration although the interpretation of the significance of the results is difficult [103]. Also the dynamics of events in utero and ex utero particularly as indicated by gene expression has received considerable attention [98] but we have to come back to the clinical observation that scarring is the outcome, not the process. In the review referred to before 'Scarless Wound Healing: Chasing the Holy Grail' [100] the authors conclude, "Achieving scarless wound healing in the adult will require not only an understanding of signalling molecules and growth factors but also a thorough understanding of lineage-specific cellular origin and function during both fetal and adult stages." With great respect for the senior author of this paper, Michael T Longaker, I would like to think that the emerging discipline of regenerative medicine is going to provide us with clinical observations that are real and stand the test of time, but, like the scarless healing achievable in the human fetus may remain a biological mystery.

We now have a story that began over a hundred years ago with the birth of a doctor who was driven to cross-fertilize his ideas with cutting edge technology in other disciplines. The result, high-definition ultrasound, opened up a whole new world of interventional obstetric care creating the reality of human fetal surgery. The ability of the human fetus to heal surgical wounds created in the second trimester without scarring was a clinical observation, not a scientific breakthrough. The failure to understand the fundamental biological mechanisms involved despite the massive allocation of time and resource from a multi-disciplinary scientific community should not diminish the energy for the quest. Rather it supports the case that the focus should return to clinical observation in the context of human regenerative medicine, the 'new reality'.

Acknowledgement The Boston Days: Paul Ehrlich introduced me to Collagen, Toni Rittenberg introduced me to the fibroblast populated collagen lattice, Rita Greco

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Part XV

Fetal Growth and Adult Life Implications

Implications of Gross IUGR in Adult Life with Respect to Some Major Diseases

38

Priyodarshi Sengupta and Niranjan Bhattacharya

Introduction

Intrauterine growth retardation is often defined as a medical phenomenon where the weight of the newborn during the gestational stage according to its sex and age are considered to be poor or less than expected according to medical and population parameters. The causes of IUGR, as it is often abbreviated its causes are still unknown and not definite although a multitude of factors can be attributed, like poor nutrition of the mother, lack of placental insufficiency resulting in diminished intrauterine fetal oxygenation, exposure to teratogens and pathogens and in some cases, molecular aberrations also termed fetal reprogramming. The long term complications of a baby born through IUGR are immense and grave like hypertension, increased risk of metabolic diseases like dyslipidemia, diabetes mellitus and cardiovascular disorders, renal impairments in some cases, improper brain and neuro-functional development and in some rare cases, onset of auto-immune diseases like rheumatoid arthritis and systemic lupus erythematosus.

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IUGR and Increased Risk of Cardiovascular Diseases, Hypertension and Atherosclerosis Conditions

There is a high rate of cardiovascular diseases, metabolic diseases and hypertension in the early stages of development related to IUGR according to different epidemiological studies. A study conducted by Cosmi and Zanardo [1] suggested that blood pressure measurements are significantly higher in IUGR children than those born under normal growth conditions. It is postulated that due to changes in the Angiotensin activity, diseases can be triggered in IUGR patients. Also hypoxia, increased sympathetic nerve activity and catecholamine production may augment the pathogenetic trigger [2].

A strong positive correlation was found between the reduced number of nephrons [3–7] and hypertension in case of sheep [8], rat [9] and humans [10]. Many animal models have shown, due to lack of proper growth of the renal system, onset of hypertension is extremely high. Studies conducted by Michael G. Ross and Marie H. Beall in rat models has proved altered gene

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expression in the rat fetal kidneys which has a direct relation with an increased propensity to adult hypertension in later life [11]. It is further suggested that the end of the second trimester till 34 weeks is the most vulnerable period during which altered renal development can result in hypertension of the neonate [4].

The aetiology of hypertension among IUGR patients are multi factorial. Another important factor leading to hypertension in IUGR affected children are the changes in the composition and structure of the extra cellular matrix which often helps in maintaining the arterial pressure [12]. The status of the ECM often changes as seen in adult rats with 8 weeks of high salt intake; Changes like increase in the aortal wall thickness, decrease in the content of collagen, elastin, smooth muscles, which are important extracellular components of the ECM [13]. This overall change of the ECM also affects the vascular changes, as observed in IUGR adults in animal models. Also the incidence of hypertension due to IUGR is observed more in male offspring [11].

The vascular endothelium is another area where several studies have shown that endothelial mediated or non mediated vasodilation is normally impaired in IUGR patients, resulting in a decrease of flow mediated dilation in the birth weight of neonates, later childhood and early adulthood [14–16]. Undernourished rats in the first 18 days of gestation also showed reduced vasoconstriction response to phenylephrine and norepipnephrine in isolateral femoral arteries [11].

Impaired growth in utero often results in endothelial dysfunction which is an early stage of atherosclerosis. This event silently develops in the neonates and often culminates in increased CVD's or an early stroke [17]. This can be detected by means of ultrasound imaging. Skilton and co-workers in the year 2005 compared intima-mediated thickness or aIMT of the aortic wall in newborn low weight infants with respect to normal weight babies and thereby developed a biomarker of atherosclerosis in infants via USG guided assistance [18]. Similarly carotid thickness intima was shown to be greater in IUGR children which was further confirmed by Crispi et al. [19]. These changes further persisted in childhood stages of IUGR born infants with increased risk for myocardial infarction and stroke. The reason behind the increase in the arterial wall thickness can be attributed to the fact that in IUGR babies there is an increased chance of metabolic reprogramming which further has a direct effect on the changes in cardiac morphology, clinical cardiac dysfunction and hypertension. These factors were also found to be independent of gestational age at delivery, lipid profile and body mass index of the infant [2].

It is postulated that cardiovascular diseases in adults are often due to quality of life as well as due to genetic factors. However a new concept has emerged regarding the onset of cardiovascular diseases which can be attributed to low birth weight especially among IUGR infants. As David Barker in 1989 [20] in Southampton confirmed a direct correlation between increased cardiovascular mortality and IUGR, it is now a well known fact also observed in rat and other animal models [21, 22] that IUGR born infants have a high susceptibility to disorders like dyslipidemia, coagulation, diabetes and hypertension [23]. This can be attributed to the high level of genetic complexity and their different epigenetic interactions and is known as the "Fetal reprogramming" where any molecular or genetic changes to the fetus in utero can result in key organ changes [22–25]. During the time of fetal development the cells undergo extensive mitotic divisions and thereby remain sensitive even to the smallest changes that can impair the structural growth and development of the fetus [23].

The proposed mechanism of IUGR induced CVD and hypertensive disorders can be related to four important factors: (a) lack of proper nutritional diet by the mother, (b) uteroplacental dysfunction, (c) exposure to harmful toxic drugs/chemicals and (d) environmental, external factors and genetic changes.

Many rat studies have confirmed that maternal low-protein diet during the gestational phase gave rise to a consistent rise in blood pressure, endothelial dysfunction, vascular dysfunction, increased ACE activity, decreased nephron numbers and increased oxidative stress in adulthood [26–32]. Also it was seen that a permanent ligation of both the uterine arteries led to hypoxia and increased

obstruction of blood flow which later had a direct implication on the onset of diabetes type 2, altered brain development and proteinuria in both the early and adult stages [33, 34]. It has been further observed in rats that with low protein diet, pulmonary and cardiac genes can get affected too.

Treatment with glucorticoid dexamethasone of pregnant rats has shown low birth weights and persistent elevation of the arterial blood pressure in both adults and offsprings [35, 36]. Also low protein diet has been found to increase the maternal glucocorticoid level due to a decrease in activity of 11 Beta-HSD2 in placenta as observed in animal models leading to low birth weights and persistent hypertension in adult life too [37]. Environmental factors like high altitude, maternal ventilation during pregnancy, can give rise to increased arterial oxygen saturation [37]. When pregnant rats were exposed to chronic hypoxia between 15 and 21 days of gestation, there was an increase in the percentage of fetal cardiac apoptosis and an increased susceptibility to high CVD, cardiac hypertrophy and ischemic reperfusion injuries [37, 38]. Maternal smoking can be also one of the most important aetiology to risks of hypertension and development of IUGR in neonates leading to fatal consequences in late adulthood [39, 40].

The fetal environment can also result in epigenetic modifications like changes in gene expression, mutation or alteration in the DNA sequences, activation or silencing of the gene expression through DNA methylation, histone modifications and micro RNAs. These alterations can have a serious effect on the onset of CVD among IUGR induced babies and in adults.

Lack of follow ups in epidemiological studies is one of the commonest obstacles faced by researchers and clinicians while assessing the relation of IUGR and low birth weights. A study which included 15,000 Swedish men and women over 50 years with a 97 % follow up, revealed a strong correlation ship between CVD and low birth weight [37, 41] whereas another study by Rich-Edwards inferred that that was a strong negative relationship between incidence of birth weights and non fatal coronary heart disease and stroke [37, 41, 42].

IUGR Effects on the Brain and Cognitive Developments

Multiple follow up studies, clinical datas and observations have shown that intra-uterine growth factor is consistently related to many disabilities related to cognitive function, cognitive skills, language, abstract reasoning and thinking, gross motor skills, memory, concentration and mood related disorders [43]. Animal studies have recently related the negative effects of chronic intra-uterine hypoxia and malnutrition to IUGR which in turn has a direct effect on the neuronal cell number restriction, cell size, lighter weight of the brain, lower neuronal DNA content as well as on the (reduced) number of functional synaptic connections [43, 44].

Further progressive deterioration of the hippocampal pyramidal neurons, loss of oligodendritic branches and reduction of the cellularity by 30 % [45] has been observed in animal models with IUGR. These losses resulted in a decrease of the overall hippocampal volume accompanied with loss of density in the granular neurons [46]. With age there have been reports of increasing neuro endocrine anomalies resulting in the modification of the hypothalamo-pituitary adrenal axis (HPA) [43]. Also recent studies pertaining to IUGR infants have observed that repeated antenatal glucocorticoid therapies and post natal corticosteroid therapies have resulted in significant reductions of the overall brain size and in the cortical gray matter volume in case of IUGR infants [47, 48].

It has been well documented that early fetal growth retardation has a direct implication on the neuronal and neurobehavioral development of the individual [49–51]. The different behavioural domains like academic skills, cognitive abilities, problem solving skills are significantly lower in cases with children born with IUGR defects. These limitations were related to the frontal lobe function which fails to develop completely in case of IUGR born babies. This can be also attributed to maternal malnutrition especially during the third trimester that is when the brain growth maintains maximum maturity and becomes more vulnerable. Fetal retardation can often lead to the

birth of babies with smaller head size. Cerebral arterial flow via Doppler findings have shown that changes in the cerebral flow can induce specific developmental abnormalities of the fetal brain which can have a profound effect on the child during the onset of adulthood [52]. Abnormal neural development in children and adults are among one of the most important consequences of intrauterine growth restriction at the time of birth.

IUGR can be normally classified into symmetric and asymmetric. In case of symmetric IUGR, the head, length and weight of the body becomes proportionally small for gestational age (SGA); it is termed as asymmetric when the head grows bigger compared to the length and weight of the body. This phenomenon is known as the "Brain Sparing effect" which is a haemodynamic effect with increased flow of blood to the brain thereby directing most of its activity towards the main vital organs [52, 53]. Children born through IUGR also show signs of restricted brain development with major reduction in the frontal lobe region and volume thereby affecting various daily skills like speech, language and creativity. According to the statistics released by the Institute of Medicine, one-third American women who have given births to babies with encephalopathies and neural tube defects, had been subject to excess nutrition where BMI >25 kg/m^2; further, more than 30 % were obese [54]. Therefore maternal nutrition has a profound effect on the outcome of the neural and brain development of the child which if not properly developed can have a debilitating effect in the later stages of adolescence and mainly impairment in cognitive skills as it has been observed among IUGR born 7 year old kids with regressive IQ [55].

Autoimmunity and IUGR

Among auto immune diseases, Systemic lupus erythromatosis and rheumatoid arthritis deserves special mention as these are the most widely studied auto immune diseases in infants, especially children who are born via IUGR method. Early life events like IUGR have long lasting effects on the immune function thereby resulting in RA and SLE [56]. IUGR is primarily linked to malnutrition of the mother and this malnutrition has a severe effect on the thymus maturation and development. One of the main functions of the pre natal and neonatal immune systems is to continuously learn the process of recognizing and destroying infections without any induction of auto immune disease. T cells of the thymus play a major role in this function and failure to form the thymus in the developmental stages or rather the formation of a small immature thymus results in the variation of the self-tolerance phenomenon [57] as seen in animal models. Normally in human IUGR conditions there is a long lasting effect on immunity including a reduced response to recall antigens from childhood vaccination stages [56].

Implications of IUGR on Metabolic Disorders and Diabetes and in Adult Life

Many of the metabolic disorders like insulin resistance, dyslipidemia or fat deposition can be attributed to IUGR, since there is an endocrine origin to changes in the hormone bioavailability during early growth and fetal development [58–61]. Abnormal levels of catecholamines, growth hormones, insulin have been observed in children born with IUGR [62, 63]. Seventy percent of the whole body glucose uptake is initiated by the skeletal muscles which is also the primary centre for insulin mediated glucose uptake apart from other organs like liver, pancreas and adipose tissues. It has been observed that hypoxia induced in the in utero environment can result in the diversion of blood flow maximum to the brain and adrenal glands thereby resulting in a baby with a higher head mass and low skeletal and body mass [64, 65] resulting in severe altered insulin resistance and other metabolic disorders. These observations tend towards a hypothesis that changes in key endocrine factors like leptin, adiponectin can often be associated with intrauterine conditions which might give rise to adult

metabolic diseases in the fetus in its early childhood stage [61].

IUGR is also found to be associated with abnormality in the fetal adipose tissue and growth apart from the fetal skeletal muscles. IUGR born babies show a remarkable reduction in the body fat mass mainly affecting the amount of adipocytes. Although total body fat reduces, increase in the visceral fat has shown to occur [66]. This results in rapid increase of distribution of centralized fat tissues even if they are not overweight [67, 68] with abdominal tissue showing hyper reactivity to catecholamines and early insulin resistance [69]. Also polymorphism of the gene peroxisome proliferative activated receptor g2 (PPARG) has been shown to impair metabolic function resulting in a case of insulin resistance leading to a high risk of Diabetes Mellitus type 2 [69–71].

Adipocytokines of the adipocyte derived hormones resembling an endocrine type of activity helps in regulating the body metabolism and homeostasis [72–74] and plays an important role in the metabolic activities in early life and might be abrogated in cases of babies with IUGR [67].

According to recent data, malnutrition of the fetus can result in obesity or obesity related disorders that might alter the secretion of important metabolic hormones like leptin and adiponectin [75]. Leptin hormone plays a central role in maintaining the functional characteristics of mass before birth and in the control of substrate utilization. IUGR is directly related to lower concentration of leptin as several studies have shown decreased placental leptin production [76] resulting in reduced fat mass and adipose tissue accumulation [77]. However other researchers have concluded higher concentration of leptin in IUGR infants, children and adults when compared with normal born babies, children or adults. This might be due to the adipocyte dysfunction associated with IUGR [78].

Further, higher concentration of leptin in IUGR, according to an older report, might be attributed to the differences in the fetal oxygenation status as leptin is highly sensitive to oxygen abundance [79] and IUGR fetuses with severe distress have shown to include significantly higher leptin concentration per kilogram of weight [77] which may lead to the pre disposition of excess fat in later life [80]. It has been observed that maternal administration of leptin results in an increase in fetal pancreatic insulin content which further gives the fetus a long term protection from type 2 Diabetes mellitus and obesity.

Adiponectin, another important and abundant cytokine found in the adipose tissue, plays an important role in modulation of glucose and lipid metabolism in insulin sensitive tissues [81]. Circulating adiponectin concentrations help in the decrease of insulin resistance states and its content is inversely proportional to the body mass and weight, unlike leptin [82]. Adiponectin is also produced in the intra uterine environment by both the placenta and the fetus [67, 83] thereby suggesting its important role in fetal growth and promotion of insulin via its insulin sensitizing mode of action [83]. Through different clinical observations among IUGR children it is believed that low adiponectin levels in IUGR infants may actually predict the subsequent development of visceral fat and insulin resistance [67]. Contradictory to this certain researches observed normal levels of adiponectin in animals and humans inspite of the fact that many are insulin resistant [67].

An interesting observation is also that the shift in insulin sensitivity appears to be sex specific as seen in case of rodents. It has been noticed that that in cases of males there is a higher rate of Insulin impairment when compared to that of females [84].

Adult IUGR human subjects having insulin resistance have demonstrated a failure to up regulate muscle GLUT 4 after insulin stimulation [85–87]. In low weight human infants a reduced expression of the transcription factors p85, p110 beta subunits of PI3k has been observed with a reduced skeletal muscle expression of the GLUT4 expression and further a blunted phosphorylation of Akt with respect to insulin infusion [88].

OXPHOS or oxidative phosphorylation is also impaired in low birth animals due to decrease in mitochondrial NOS [89]. This results in low birth weights and decreased ATP and insulin production [90, 91]. It has been reported that in humans

with low birth weights, an increase in glycolytic fibers also precedes insulin resistance [92].

Additionally high amounts of saturated fatty acids have also been related to insulin resistance [62]. Any alteration in fatty acid metabolism and oxidation impairs the ability of the skeletal muscle metabolic activities and is shown to have a direct effect on IUGR [89, 93]. This results in the accumulation of lipid metabolites in the skeletal structure which is linked with increase in stress induced protein kinases C (pkC), c-JNK, inhibition of NFkappa Beta kinase, further leading to insulin impairment.

Another important reason for the later onset of diabetes disorders as found in IUGR born individuals can be related to the fact that due to increased hepatic glucogenesis observed in rats [11], it can often precede the development of hyperglycaemia resulting in the resistance to insulin effects. Up regulation of PPAR (peroxisome proliferator-activated) gamma co-activator 1 alpha, mRNA regulator of glucose-6-phosphate and other gluconeogenesis enzymes may also alter the hepatic glucose production resulting in intracellular cell signalling changes and high glucose production which might overcome the effect of insulin [11].

Conclusion

From the above review it can be said that malnutrition is one of the major factors behind the cause of IUGR in rodent model though the problem is not so simple in the human system. There was an observation made during the second world war that the weight of the fetus upto 24 weeks showed no statistical difference in mothers belonging to the social upper or elite class starvation induced mothers with progressive malnutrition, who were deprived by the invading army.

IUGR has severe implications from the post natal period to adult life of an individual and can be fatal sometimes. With the development and advent of modern diagnostic techniques like three-dimensional USG, MRI and other invasive techniques, IUGR can be detected at a much earlier stage in the pregnant woman and thereby necessary steps can be taken to prevent further deterioration. Normally fetuses have an alternative mechanism to combat nutritional restrictions and severe fetal hypoxia but often an increase of such high risk factors can severely impair the growth and function of the fetus in utero and can have major implications in future growth and development.

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Part XVI

Understanding Fetal Growth from the Perspective of Alternative Medicine

39 Growth and Development of the Human Fetus Up to the Second Trimester: A Perspective of Traditional Chinese Medicine

Mursheed Ali and Niranjan Bhattacharya

Introduction

Traditional ways of practicing medicines during pregnancy are still prevalent in many nations, for example, Turkey [1], African countries [2], Thailand [3] and China. There is a likelihood that following traditional practices during pregnancy has both medicinal and harmful [1, 4] outcomes. With regard to pregnancy, there are no significant differences among distinctive socioeconomic groups. Some women may have doubts about traditional customs, but still take after the advice of the older generation [3], while other women may modify their social behavior [4, 5] to defend their unborn baby from danger [5].

In Chinese culture, there are traditional pregnancy confinements to protect the fetus from "malign influences" and to prevent pregnancy-related issues, for example, miscarriage of the fetus, stillbirth, death of the mother, and defects in the baby [5]. The fundamental ideas of these limitations originate from the notion of yin and yang in Traditional Chinese Medicine (TCM), since 2000 BC [6, 7]. TCM originates from the Taoist theory of an individual's harmony with the universe. As per this hypothesis, the human body is similar to the universe, and can be divided into a positive force (yang) and a negative force (yin) which are integral to one another. Health is considered to be a balance between the yin and the yang [8]. TCM has particular key ideas, including the qi (vital energy), which is accepted to circle along 14 channels or meridians, 12 of which impact are affected by the major internal organs. The qi is kept in balance by the double polarities of yin and yang [8], and the disturbance of the qi in a pregnant woman is thought to bring miscarriage or fetal malformation [9].

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Chinese Medicines for Pregnancy and Their Impact on the Developing Fetus

Chinese medicine incorporates a few separate medications which are connected in an unexpected way; however they all take into account the same assumptions and insights of the nature of the human body. Fundamental therapeutic strategies [10] incorporate acupuncture, Chinese

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medicines (applying traditional medications, for the most part derived from herbs, animals, and minerals, to cure ailment and maintain good health), nourishment treatment (diet to meet specific nourishment requirements and herbs to adjust internal bodily needs), Qi Gong (advancing health through breathing and meditation exercise), Tai Chi (the development of muscles and exercise of related joints), Tui Na (back rubs to stimulate the meridians and enhance the blood flow), Cupping (relieving blood stasis and pain by creating vacuums on the body surface), Die Da (bone setting) and Gua Sha (scaling the skin to stimulate specific acu-points until mild to moderate subcutaneous haemorrhage occurs) [11, 12].

For centuries, Chinese medicines have been generally used to mitigate numerous side effects and to treat complications during pregnancy [13]. Some Chinese medicines are prescribed for utilization during pregnancy and particular herbs are guaranteed to be safe and can be very viable. It has been utilized to prevent miscarriage and preterm labor, and to manage common colds, low back pain, low fetal weight, placenta previa, uterine fibroid and other obstetric issues [14]. More than 9 % of pregnant women devour herbal medicines or supplements around the world. The commonness of utilization in pregnant women is high in the Pacific nations; for instance, 78.7 % in Japan [15], 36 % in Australia [16], 32 % in Mainland China [17] and 24 % in Taiwan [18]. Chinese medicines are frequently utilized in threatened miscarriage to prevent pregnancy loss [19–22].

Lu Li reported on threatened miscarriages, that if there was an unevenness in the mother's system (however, this was not applicable in case of genetic issues with the fetus), it could frequently be overcome with the use of herbs and treatment with moxa or acupuncture [23]. The method to be utilized and the strategies to follow should be discussed early in the pregnancy so that appropriate steps can be taken should bleeding, fetal agitation, or early contractions occur. It is critical to note that most instances of early miscarriage (sometimes called spontaneous abortion) are not identified with an irregularity in the mother's systems. Later in the pregnancy, shortcomings in the mother's system or fetal development not considered to be normal. There is a specific herb formula, called Tang-kuei and Peony Formula (Dang Gui Shao Yao San), which aims at avoiding miscarriage; however, the formula is expected to be utilized primarily as an everyday preventive treatment instead of an emergency treatment. Extensive testing in the oriental countries has shown this formula to be safe and successful; modified versions of this formula, such as Tang-kuei Formula (Dang Gui San), are utilized to address particular concerns and are likely to be equally safe and efficacious. Other formulas, for example, "A Tai Yin" (which implies Peaceful Fetus Formula) and "Shou Tai Wan" (which implies Fetus Longevity Pill) are prominent cures in China for utilization during the latter part of pregnancy.

Jiang Hong assessed the impact of Jinye Baidu Granule (JYBDG), a traditional Chinese medicine compound prescription, on fetal development and maternal active human cytomegalovirus infection [24]. As a result of active human cytomegalovirus infection (HCMV), mother-fetus vertical transmission may cause abortion, malformation, fetal intrauterine death, development retardation, and in addition long haul sequelae, such as, damage of hearing or vision and intelligent disturbance of neonates. It has been noted that active HCMV infection rate in pregnant women is around 11.2 % [25], which causes obstruction to the growth and development of the fetus and neonates; so far, few effective treatments have been found. Therefore, A study was conducted in 3,187 pregnant women with active HCMV infection using Jinye Baidu Granule, JYBDG, a traditional Chinese medicine compound. mRNA was screened by enzyme linked immunoabsorbent examine (ELISA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR). The growth and development of the fetus were also investigated to examine whether the Chinese medicine had a remedial role.

Traditional Chinese Pregnancy Restrictions and Health-Related Quality of Life

Health related quality of life is an undeniably vital issue. It is an expansive, multifactoral development that surveys the level of prosperity

felt by people and can fluctuate as a result of diverse social impacts [26]. Health related quality of life is one part of this development [26], and is related to the impact of an individual's health status on his or her subjective physical, mental, and social wellbeing. It incorporates both physical and mental areas, which has concentrated on particular issues, for example, an individual's encounters, convictions, desires and observations [27]. During pregnancy, hormonal and organ-particular changes adjust affect as well as mental wellbeing. As a result, the impression of quality of life may change [28]. Hypothetically, pregnancy confinements may be seen as having both positive and negative effects on health related quality of life. These limitations may be connected to antenatal health practices that persuade women to have a positive disposition toward pregnancy, to get ready for child rearing, and to recognize this life move [29]. One study found that a more inspired state of mind was connected with a better quality of life [30]. Then again, pregnancy may limit women's physical movement and eating regimen; accordingly, women lose some of their flexibility [4]. One study found that health related quality of life was lower in pregnant than in non-pregnant women [31]. Thus, pregnancy can compromise physical and mental function [4]. One study found that taking traditional Chinese medicines during the perinatal period may have a constructive outcome on women's health related quality of life; however. The persistent use of these medicines may have a contrary impact [32].

Pregnancy is a vital stage in life for some women. The physiological changes caused by fetal development (including postnatal adjustment) and birth of a baby influences maternal health. A person's constitution, as per the hypotheses of Traditional Chinese Medicine (TCM), is characterized by intrinsic hereditary and obtained elements, which incorporate appearance, physical function and mental status [36]. There are complex morphological, auxiliary, physical and psychological dimensions. These qualities can affect an individual's susceptibility to disease and pathological tendencies [4]. Chinese medicine has four diagnostic strategies (counting examination, auscultation/olfaction, history taking and palpation) to diagnose, for instance, the 'Qi vacuity pattern' [37]. Humans are complex organisms who display continuous changes in their constitutions following changes in age, diet and living environment [38]. TCM claim that giving birth causes an insufficiency in qi and blood causing women to become physically weak. Consequently, postnatal women are inclined to blood stasis and illness when exposed to either cold or dampness. Furthermore, insufficiency in qi/blood renders postnatal women susceptible to common cold and other illnesses [39]. Therefore, constitutional changes during the perinatal period are especially imperative. During pregnancy, minor constitutional changes can lead to the replenishment of qi and blood, which guarantee proper fetal development [38]. An ancient Chinese colloquialism states that women are inclined to fear cold during early pregnancy (which is sometimes uncomfortably manifestated), but have adverse responses to heat in the later pregnant stages. This suggests that the constitution experiences considerable changes during the various phases of pregnancy. Wang [40] reported significant differences in constitution before and after pregnancy, observing that the average scores from a constitutional scale increased during pregnancy.

Traditional Chinese Medication is presently acknowledged as a standard form of medical care all over East Asia and is viewed as a corresponding or optional solution in the Western world. Chinese herbal medicines have been utilized to prevent abortion and preterm labor and to deal with common cold, low back pain, placenta previa, fetal development confinement and other obstetric issues [14]. Some Chinese herbal medicines are commonly utilized during pregnancy and they are asserted to be "safe" and "successful" [33]. Unlike those pharmaceutical medications not suggested for utilization during pregnancy due to known adverse or teratogenic impacts in animal studies and/or clinical studies, Chinese herbal medicines are endorsed based on individual involvement in clinical practices; but, there are no sufficient statistics or scientific information with respect to the adverse maternal and perinatal

effects of Chinese medicine. Therefore, the actual impact of TCM on pregnancy, embryo fetal development, prenatal and post-natal growth is yet unknown. The procedure for testing herbal medicines in humans has not yet been established. Before clinical studies can be attempted in humans, the initial phase is to develop guidelines on the assessment of quality, efficacy and safety of herbal medicines through testing in animals. There are traditional convictions that certain sorts of Traditional Chinese Medicines (TCMs) are valuable in pregnancy [34]. However, the adequacy of most TCMs is largely unproven and its safety has yet to be established.

Maternal and Fetal Safety Concerns of Chinese Medicines

Chinese pharmaceuticals are normally respected by people as safe [35]. Albeit there is no scientific basis for the safety claim, around 30–50 % of pregnant women use it to maintain good health and decrease the requirement for Western medicine [7]. Chinese medicines have been historically used in the cultural context, and there is no sufficient record with respect to any adverse impacts in utilizing these herbs. There is no data on how safe the medicines are during pregnancy and if there are any antagonistic impact of the medicines on embryo-fetal development. More than 2,000 Chinese medicines and prepared slices of Chinese crude drugs, vegetable oil, fats and extracts, patented Chinese traditional medicines, and single ingredients of Chinese crude drug and preparations are recorded in Chinese Pharmacopoeia [41]. At present, there are more than 300 Chinese medications which are regularly utilized during pregnancy as a part of daily clinical practice; approximately 200 are applied for promoting maternal and fetal wellbeing.

Conclusion

Chinese medicines should conform to the same modern pharmacological standards as western medicine. The active elements of the Chinese medicines are likewise chemicals that are similar to prescription pharmaceuticals. Chinese medicines are not free of danger; they are similar to western pharmaceutical medicines and have potential to cause antagonistic impacts. Chinese medicines may be beneficial, yet might likewise antagonistically influence both mothers and fetuses in utero, and may harm the fetus specifically. Until more dependable and scientific information is available, clinicians ought to consider both the dangers and advantages before prescribing Chinese solutions to pregnant ladies. More deliberate examination of the safety implications of the use of Chinese medicines in animals is suggested, and more studies with clinical trials in humans are also needed to ensure the clinical safety and usefulness of TCM.

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Perspectives of Tribal Medicine on the First 6 Months of Pregnancy

40

Priyodarshi Sengupta, Dibyendu Bandhopadhyay, and Niranjan Bhattacharya

Introduction

Tribal medicine is essentially a very age old medicine without recognition among the modern scientific community due to its lack of presence in the scientific fraternity which can be attributed to the fact that most tribal medicines lack advertisement and are secluded as they are only used by a fraction of the people across the globe in various tribal areas of Africa, India, North America, Australia, New Zealand, Pakistan and Central Asia. Traditional remedies form an essential and important part of the cultural and religious life of tribals. Tribal medicines are mainly herbs in nature just like in the case of Ayurveda, homeopathy, traditional Chinese medicine and Unani system of medicine. Nearly half the population globally relies on herbal medicine as do many tribals [1]. A wide range of herbs are normally included in many forms of medicine during various stages of pregnancy and to regulate the menstrual cycle [2].

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Tribal Medicine and Methodologies in 1st and 2nd Trimester of Pregnancy

Tribal communities are often forest dwellers and their medicinal practices have often been termed as 'traditional health care system' over the years. Most of the healing systems of traditional medicine are based on faith healing which is often equated to as confidence building according to the modern system of medicine. Magical spells play an important role in tribal medicine [3].

Most tribal people in India believe that at the time of pregnancy consumption of alcohol can cause deleterious effects in the body. It has been claimed that a certain group of tribals in the Indian state of Chhattisgarh can guess the sex of the baby by just observing the features of a pregnant mother which includes measuring the circumference of the abdomen of the mother mainly during the late 2nd trimester of development. According to them if the circumference of the stomach is large then the fetus is a girl and if she looks thin then it s a boy. Also during the second trimester if the pregnant woman feels the movement of the child inside her abdomen then it can be said that the fetus is a female child.

A certain sect of a tribal community in Assam known as the Karbis, use herbal products during the various stages of pregnancy and to treat various gynecological conditions. To carry out abortion during the 1st or 2nd trimester Ceylon Leadwort, a herbaceous plant with glabrous and erect stem which according to the folklore

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resembles the size of the penis is tied around the thigh region with a long thread and then inserted inside the vagina to initiate the process of abortion; normally it is kept tied for a day or two. Also it has been reported from tribal groups in the Indian state of Gujarat that root paste of the plant is used in the vagina for increasing the chance of abortion [4]. It is also reported that ground root mixed with a little amount of sugar if taken orally can be effective in inducing permanent sterility [5].

Concept of Pregnancy among Different Tribes

The Cholanaickan tribes in Kerala believe that the initiation of pregnancy is induced by the sexual union of female and the male where the male is regarded as the progenitor. According to the folklore of these tribes it is suspected that pregnancy which is also referred to as oddalayarathu happens as the young married woman misses her periods. Further according to these tribes, pregnancy lasts for ten lunar months and the sexual union up to the seventh month of the last trimester. During the 1st and 2nd trimester food acts as the principal medicine to these tribes where roots and tubers are regarded as roughage foods and need to be avoided. Other holistic approaches during the 1st and 2nd trimester are followed in order to keep the fetus and the mother healthy; these are massage of the abdomen with coconut oil, magic spells and spiritualism. This also continues to the last trimester and before the delivery of the baby the intensity of such rituals increases. The pregnant women during the early stages of pregnancy can be allowed to accompany men in the forest for gathering and hunting foods. Husbands, on the other hand, after the end of second trimester, are not allowed to come near to the woman [6].

Like the Ayurvedic, Unani, Chinese and Homeopathy system of medicine diet also plays a very integral and major role in maintaining a safe pregnancy period.

According to North American tribes, including the Apaches, pregnancy is a very laborious and time consuming process where the blood (semen) from a man enters a woman's system during coitus.

This male blood (semen) is opposed by the woman time and again so that repeated love is made in order to ensure the sufficient seeding of the baby and to overcome the resistance from the mother. Other tribes like the Hopi tribes believed that continuous coitus was necessary for pregnancy. Other tribes, on the other hand, abstained from sex thinking that sex during pregnancy might give birth to a baby filled with filth [7].

Among a certain tribe in South Africa, herbs are used during pregnancy orally on a regular and normal basis as it is thought that doing such will help in cleaning the womb of the mother as the herb functions as a cleansing tonic [8, 9]. It is also thought that practicing such methods it will facilitate easy delivery and protect the new born child from evil [10].

Herbs play an important role in tribal medicine. For example, plumbaginaceae herb, used by the tribal people of Boudh district in Orissa, functions as an abortifaciant by inducing abortion in the tribal mother within 3 months of pregnancy [11], whereas in the case of the various tribes of West Bengal, the abortifacient herbs used are amaranthaceae, bromeliaceae. These herbs help in inducing abortion during the first 3–4 months of gestation also [12]. Also herbs like Acantheceae can induce early abortion in the mother during the first trimester itself. Ebenaceae, another herb, is used in the second trimester to cure anaemia in tribal mothers [11].

Although tribal medicine lays a special emphasis on food as an important medicine because it provides nutritional status to the fetus in the early pregnancy months, it has been found that 90 % female Kannikar tribes in India showed anaemia, 30 % suffering from Vitamin A deficiency and 10 % from niacin deficiency [13, 14]. This trend was also common in case of female tribes from the Bastar district in India. In general tribal medicine can be defined as that branch of medicine which is still hugely unexplored and is a diversified form of medicine based on the ancient traditions of that country, like in India where most tribal medicines are based on the herbs and principles of Ayurved.

Moreover the course of treatment and standard of life maintained by different tribes in

India are also based on the principles of Ayurved and the Unani form of medicine where spiritualism, holistic measures and approaches and diet as the main course of medicine during the first 6 months of pregnancy, play an important role. Serious shortcomings of scientific explanations behind the many forms of tribal medicine and the lack of understanding of the different phases of pregnancy from the scientific point among the tribal people really makes it very difficult for scientists, public health workers and researchers to extract data regarding the types of medicines used in the first and second trimesters of fetal development. Also diversification of the tribes and their knowledge is another important criteria in making it impossible to come to a single basic approach of tribal medicine during the first 6 months of pregnancy. For example, a Maori tribal or an Apache from North America and the Kannikar tribes from Maharashtra might share a common philosophy in pregnancy based tribal medicine but their approach in applying this thought might be different and can be extremely diverse in the form of medicines they use; for instance, the Kannikar tribes would prefer the use of herbs more than the Maori or the Apaches.

Conclusion

With the emergence of more efficient healthcare access and providers, tribal medicine is slowly becoming a thing of the past with more number of tribal communities embracing and accepting the modern standard care of health and medicine. Also governments across the globe through different awareness and initiatives are starting to provide easy and free healthcare access to the different tribal communities. In India it is mandatory for government health care practitioners to spend some time in rural and tribal areas. One can see such initiatives taken in the Andaman and Nicobar islands for Jarwas, an indigenous ethnic tribal community residing in the Nicobar islands for more than 2000 years thereby resulting in a slow extinction of many of the indigenous health care practices that were followed by them over the years.

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General Perspectives and Applications of Unani Medicine in Pregnancy Up to the 2nd Trimester

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Introduction

Unani Medicine or Graeco Arabic Medicine or the well versant term of Unani Tibb is an ancient traditional system of Medicine that links itself to the Middle East and South Asian Countries especially Yunan (Greece), Persia, Syria and India. Though Martin Levey in 1952 produced a report that justified Babylon to be the earliest centre of medicine [1], the concept of health and healing is much more primitive in origin. The Tibbi system of medicine is a rich storehouse of principles and philosophies of medicine which if understood in its proper perspective can prove of immense value. However, since all Tibbi literature is in the language and terminology of the contemporary sciences of the Middle ages (Persian, Arabic, Greek, Urdu etc.) it is not understood, or is rather inaccessible to modern scientists. Thus this system of medicine has been labelled as traditional and unscientific, though it has rendered service to millions of people for thousands of years and is the mother of the modern system of medicine. Hippocrates (460–337 BC), also known as the Father of Medicine was a Unani Tabib (physician). Other eminent Unani physicians include:

- Asclepius (also known as the God of Health in the Unani System) [2].
- Aristotle.
- Joannes Grammaticus.
- Herophilus (also known as the Father of Anatomy in the Unani System) [3].
- Galen.

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- Geber (also known as the Father of Chemistry in the Unani System) [4].
- Rhazes.
- Haly Abbas.
- Juhannitus.
- RabbanTabri.
- Thabit Ibn Qurrah.
- Yaqub Ibn Ishaque.
- Abu SahlMasihi.
- Avicenna.
- Abulcasis.
- JesuHaly.
- Hazen, and many more.

Methods of Unani System of Medicine

The concept of Unani system of medicine is primarily based on prevention of disease and the well being of the individual by means of improving their standard of living and quality of life by maintaining a healthy diet and healthy lifestyle especially during pregnancy as it believed that the well being of the mother is related to the well being of the baby inside her womb. However like other principles of medicine Unani also lays emphasis on curative or pharmacotherapeutic approaches. The treatment guidelines of the Unani system of medicine is based on two important principles, namely, the preventive and curative principles.

The preventive principles are further based on the different elements of nature like the atmosphere, air, water, temperature, the mental and emotional condition of the individual, dietary intake like food and drinks, amount of stress in life and how well is the sleep and wakefulness of the mother including the movement and repose of the body.

The curative principles are based on curative regimen therapies like cupping or Majamat where the application of a glass vessel exhausted by heat is done to the skin, Diaphoresis or Tareeq which helps in lowering the body temperature and cleaning of morbid materials through the skin pores. Other methods include leeching by which toxic blood is released from the body, therapeutic bath in a hot water bath is quite common in Turkey for cleansing the body and provides beneficial support to the skin and prevents humoral diseases. Blood letting or Fasd which involves a small cut at the affected area and allowing the blood to drain out; Diuresis, also termed as Adrar-e-Bowl to cure heart, cardiac, lung and liver ailments; massage or Dalak to tone up the muscles and the nerves along with cauterization performed by a hot metal, or corrosive medicines to remove pathogens have been successfully practiced in Unani over the years. Diet therapies and Pharmacotherapy including dosage timings, dosage route, forms and shape of the drugs also form an important constituent of the curative mechanisms under Unani mode of therapy.

Unani system is very similar to the ayurvedic system of medicine in terms of its principles. Both systems of medicine, Ayurved and Unani, consider natural elements like air, water, earth and fire as key elements and any imbalance in these key elements can result in the imbalance of the nature of the human body leading to a disease state. Therefore in order to treat such imbalances herbal remedies are often prescribed in order to rebalance these elements within the human body and fight disease conditions [5].

Like Ayurveda, in the Unani system of medicine it is postulated that if treatment is possible by diet therapy then there is no use of drugs in such cases as diet plays an important and basic role in maintaining a healthy body. Certain herbs which are used as essential ingredients of cooking in countries like India, Pakistan, Bangladesh, Sri Lanka including most western nations and the Middle East are used extensively in ayurvedic and Unani medicine too. Cloves for example are used as carminative to increase the hydrochloric acid in the stomach, thereby helping to increase the peristalsis effect. The essential oil extracted from clove is also used to alleviate pain and healing in Unani medicine. It is also used for analgesic, anesthetic, antidotal and antiperspirant purposes. Coriander, another essentiality in Asian cooking, is used for treating disorders of the digestive, respiratory and urinary system, diabetes and loss of appetite. Bay leaves have important properties in controlling flatulence and relieving the stomach of indigestion. It is also used for sprains, bruises and skin rashes. Powdered seeds can be given to young girls after menarche [6].

However if the shift of the body from normal health condition is consider to be great and can

carry the risk of affecting the body to a greater extent, then diet therapy alone is not sufficient. Pharmacotherapy (Ilaj bid Dawa), like the principles of modern medicine, is also important. These drugs can be either herbal type, mineral type or maybe even derived from animals. In Unani, single drugs or their combinations in raw forms are preferred over compound formulations. Most of the drugs applied in the raw form are natural as it is believed that naturally occurring drugs are a symbol of life and have minimal side effects.

However the safety of the drug composition is also given importance in Unani medicine where drugs which are toxic in its crude forms are processed and purified. In Unani medicine, compound formulations are also used for treating various complex and chronic disorders since emphasis is laid on a particular temperament of the individual [7].

Principles of Unani System

The Unani system states that 'Tabiyat' (physis-nature) is the supreme planner of our body. It is a natural power- a prime mover which when exists in a body, becomes cause direct or cause proximate for its active motion or rest. Thus 'Al Umur Al Tabaiyah' [8] deals with the nature (Tabiyat) of a thing (Human Body) which is comprised of seven principles-

- Al-Arkan or Al-Anasir (Elements)
- Al-Mizaj (Temperament)
- Al-Akhlat (Humours or Body fluids)
- Al-Aza (Organs or Members)
- Al-Arwah (Pneuma or Vital spirit)
- Al-Quwa (Faculties or Powers)
- Al-Afal (Functions)

In addition to the above seven UmurTabaiyah, there are AsbabSittahZaruriyah [9] (Six Essential causes) that too influence the human body with respect to preservation of health or causation of disease. They are

- Al Hawawa'lMuhit (Atmospheric Air)
- Al Makulwa'lMashrub (Food and Drinks)
- Al Harkatwa'lSukun al Badaniyan (Physical Body Movement and Repose)
- AlHarkatwa'lSukun al Nafsaniyan (Mental or Psychic Movement and Repose)
- Al Naumwa'lYaqzah (Sleep and Wakefulness)
- Al Istifraghwa'lIhtibas (Evacuation and Retention)

The above six factors essentially influence each and every human being and likewise there are Asbabghayrzaruriyah (non- essential causes) which do not influence each and every human being, but may influence some, like habitat, profession, sex, social factors etc.

The term physiology also originated from the Greek root 'Physiologikos' meaning discourse or knowledge of nature or Tabiyat [10]. Thus Umurtabaiyah and Physiology are synonymous and both deal with the body and its natural functions.

The study of modern physiology begins with the 'Cell' that is regarded as the structural and functional unit of the body. Then the tissues (AlAza al-Mufradah) are studied. Thereafter comes organs (AlAzaal Murakabbah) and the systems. But in Tibb, contrary to the modern concept of cells, the lowest level of organization of the human body and other creations is the Unsur (element) or the atom (JuzLayataza). About 2500 years back, Greek Scholar Democritus and his pupils thought that all matter is made up of small indivisible units called atoma (atoms) [11]. They associate in different ways to constitute Akhlat (humour).

Now the concept is these Arkan (elements) come into Imtizaj (intermixture or chemical combinations) and produce a uniform state or state of equilibrium for each Anasiror Arkan (element) and thus this new state of matter is called Mizaj (temperament).

The humoral theory postulated by Hippocrates (460BC) in his famous book *Tabiyat al Insan* (Human nature) states that "The body contains four (major kinds of) humours, Dam (blood), Balgham (phlegm), Safra (yellow bile) and Sauda (black bile); a right proportion, according to quality and quantity, and mixing of which (homeostasis) constitutes health and unright proportion and irregular distribution, according to their quantity and quality constitutes disease" [12].

Ali Ibn al-Abbas [13] says Akhlat (humours) are the proximate principles for the human body. But the more proximate are the AzaBasitah

(cells and tissues) which are composed of Akhlat, and Aza Aliyah (organs), which are composed of AzaBasitah. Ibn Sina also has the same view [14].

The concept of Quwa (faculties, power) is unique in Tibb. The Quwa is that property of the body through which life is manifested. It provides the basis of many bodily functions. These Quwa (faculties) are specific for a tissue or organ. For example, the Brain can only perform functions (Afal) pertaining to Quwat e Nafsaniyan (nervous system) and cannot perform the functions (Afal) of the heart, liver or kidney.

Thus the organs (Aza), faculties (Quwa) and functions (Afal) are interrelated and interdependent. For each function there is a Quwat (faculty or power) and where there is Quwat, there are Afaal. (Nafis) [15].

Concepts of Unani Medicine in Terms of Modern Medicine

The concept of modern allopathic medicine is based largely on the disease and suffering of the patient. It is based largely on curing and treating the individual whereas Unani and Ayurveda are based on the concept of prevention rather than cure through different holistic and natural methods. However with increasing evidence that many diseases actually arise due to the ingestion of materials that cause abnormal reactions to the body, more and more emphasis is focusing on diet control and therapies, including at the time of pregnancy. Modern therapeutic approaches come at a cost with adverse drug reactions; so to minimize such reactions often traditional complementary and alternative medicines, also known as (TCAM), play an important role. Various Unani formulations include rationality of using various medicinal plants, animal products, methods of preservation and administration, indications and contra indications, avoidance and abstinence of certain diets (Parhez), complete drug profile, drug effect and kinetics, adverse drug interactions, drug to drug and drug food interactions, thus making it acceptable and on lines with modern systems of medicine [16].

In Unani formulations of drugs, proper and logical reasoning in the preparations of drugs are followed thus making it a very scientific field of medicine. The above methods and considerations are based on lines of the principles of modern medicine where correctives (Muslehat) to drugs have been in use for a long time so to as negate the undesirable effects of different drugs to the body, which otherwise in the normal combined prescription of both single and combined drugs can produce unwarranted effects. In spite of the fact that crude toxic drugs are purified in many ways (Tadbir) these drugs may still have side effects (muzarrat) and the sole responsibility of prescribing these drugs depends on the well informed and experienced physician so as to avoid any unnecessary adverse events [16].

Another characteristic of Unani medicine is the use of wide varieties of herbal drugs which are different in different countries. These herbal drugs have a profound effect on the physiology of the body and mechanism of different herbal drugs varies from country to country and within its genera, thereby increasing the potential chances of toxic diversities. In order to safely monitor such use of herbal drugs, an international program has already been undertaken by the WHO Collaborating Centre for International Drug Monitoring of Herbal medicines [17].

Fetal Development in Unani Medicine

After copulation when the sperm enters the uterus, mani (germ cells) rests for a while there, thus causing pregnancy (Halat-e-Hamal) [18].

According to Hippocrates [19] Quwat-e-Muwallidah (generative faculty) extracts semen material from every organ of the body, thus it is capable of forming every organ from which it is derived. Conception occurs when both mani (sperm and ovum) combine together resulting in the formation of Nutfa (zygote). Since both matter (element) and quality (temperament) are present in mani, this mixing produces a new temperament (Mizaj). If this temperament lies within human limits, conception occurs and the

zygote gets implanted in the uterus for further development. If the uterine environment and zygote interact and exceed the limit of human temperament in total, abortion occurs.

At first instance the Nutfa gets frothy due to Quwat-e-Musawirah (formative faculty) which is an inherent property of the uterus. The sperm already has Ruh-e-Nafsaniyah (psychic pneuma) and Ruh-e-Haiwaniyah (vital pneuma). These inherent properties of sperm get stimulated by the Quwat-e-Musawirah of the uterus and as a consequence each Ruh leads to genesis of their specific organ (Ruh-e-Nafsaniyah leads to genesis of cardiovascular system and Ruh-e-Tabaiyah leads to the formation of the gastrointestinal system) [20].

Ruh is a gaseous substance obtained from the inspired air (oxygen); it helps in all metabolic activities of the body. It forms the AkhlatLatifa (fine humours) to produce all kinds of Quwa (powers) and HararatGhariziyah (innate heat); it is the source of vitality for all organs [21].

According to Ibn Nafis [22] Quwat-e-Musawirah is that quwat which gives shape to each part of mani (sperm and ovum) which is required by that particular species. Giving the shape means it produces lines in the organs, forms cavities and depressions and performs other functions.

Al-Abbas [23] is also of the same opinion. Thus, Quwat-e-Musawirah controls [24]:

- Male and female sexual powers and functions of the heart copulation (potency)
- Fertilization of ovum
- Transplantation of ovum
- Cleavage and differentiation of zygote
- Formation of membranes and fetal parts
- Development of fetus
- Parturition

To elaborate, the frothy product formed at the time of conception reaches a place in the uterus where there is a favourable condition for the formation of the heart of the foetus. From the point where the heart generates, two prominences, one on its right and the other on its upper side appear. Thereafter they separate out forming a zone for the heart form the right prominence and the liver form the left prominence [25].

Subsequently the frothy product is filled with whitish blood (SafediMaelkhoon) and then an orifice is formed by a Pneuma (NafakhRehi) so the conceptus can get its nutrition, that is, blood (dam) and oxygen (ruh) form the uterus. Thus the umbilicus (naaf) is formed. So the generation of heart, liver and brain occurs prior to the umbilicus and the umbilical cord.

According to Hippocrates, the first organ formed in the developing embryo is the brain and two eyes. He justifies it by citing the example of an egg where these two are the first organs to be formed, whereas Aristotle claims that the heart is the first organ to be formed because it the centre of both life and Hararat-e-Ghariziyah (innate heat) [26].

At the time of conception the amniotic membrane (Gisha-e Janini), formed due to the movement of ovum and sperm, provides protection and nourishment to the conceptus till the requirement of nourishment is less and the membrane is still thin.

Ruh-e-Haiwani (vital pneuma) and Hararat-e-Ghariziyah from the heart goes to the other viscera and the Quwat-e-Musawirah (formative faculty) already present in them, get activated. As a consequence, the chief organs of the system get ready to function separately with the help of their supportive structures. For example, the brain starts functioning separately along with the peripheral nerves.

During the first week of conception, the mani gets frothy and Quwat-e-Musawirah starts performing its function in the zygote. After 3 days of this period (on ninth day of conception) with the help of the uterus, cleavage appears in the zygote. On the 15th day blood enters the zygote and a mass of flesh forms. From this fleshy mass, the heart, brain and liver are consequently formed which separate out and the spaces between them are filled with mucus. On the 34th day, the head gets differentiated from the shoulder and becomes prominent. At the same time, the ribs and the abdominal cavity are formed [27].

The temperaments (Mizaj) of maternal and paternal germ cells are the determining factors of the sex of the embryo. As in Tibb, sex is a temperamental character, which if hot, the embryo would definitely grow into a male. The reason for more

expansion of the organs in males than females is this higher level of heat, thus giving a strong physique to the male compared to a female of the same age. After the embryonic temperament has been established, Quwat-e-Mughayyirahula (primary transformative power) works in the embryo and prepares its different parts to acquire the properties of different organs. It differentiates parts of the embryo into organ specific temperaments and consistencies, whereas Quwat-e-Musawirah (formative faculty) has the specific ability to prepare the specific dimension and shape of the organ. The failure of Quwat-e-Mughayyirahula results in absence of organs, whereas failure of Quwat-e-Musawirah results in structural anomalies though the organ exists. These two Quwat work with ample heat and its deficiency in early developmental life can result in death. One such condition is a mole (Hydatidiform mole) [19].

The functions of Quwat-e-Mughayyirahula stops when every part of the embryo is ready to form a specific organ, accepting the action of Quwat-e-Musawirah. In early stages of embryonal development, maternal Quwat-e-Haiwaniyah (vital faculties) give the embryo the ability to accept the functions of Quwat-e-Tabaiyah (natural faculties), but after sometime the embryo acquires it from the development of its own heart. Now the fetus is ready to accept the functions of Quwat-e-Tabaiyah and Quwat-e-Nafsaniyah (psychic or mental faculties).

The uterus along with providing protection and nourishment to the fetus, also provides the site of action to Quwat-e-Mughayyirahula and Quwat-e-Musawirah as neither of these function outside the uterine cavity or in post natal life. As congenital abnormalities are a result of malfunctioning of Quwat-e-Musawirah, Quwat-e-Mughayyirah and temperament, all these need to be modified in order to bring back the healthy state. Temperamental change though can be applied in that case, but as stated earlier, these two Quwats exist only in utero posing difficulty in permanent cure.

Quwat-e-Masika (retaining faculty) helps the fetus to remain in the uterus while its weakness strengthens Quwat-e-Dafiyah (expelling faculty), thus causing its expulsion.

Pharmacovigilance and Safety of Unani Drugs

During the first 2 months of pregnancy Unani medicine stresses on the well being of the mother and the fetus by stressing on diet therapy and very much less on pharmacotherapy. Apart from diet therapy other important considerations like the emotional state of the mother, sanitation and hygiene of the mother and her surroundings, standard and quality of life are all important factors which play a dominant role in the well being of the child and the mother during the first and second trimesters of pregnancy. Unani drugs are basically based on temperament, potency and efficiency. Higher the degree, higher is its adverse effect. Temperament or Mizaj is an important constituent of the Unani drugs and is based on 3° like cold, hot and dry.

During pregnancy if the mother does not respond well to the above concepts of Unani therapy, substitutes' or Abad al Adina can be used only. If a drug or therapy is substituted by some other means of treatment or medicine then that new medicine or therapy should be unique and equally or more effective than the previous one, thus rationalizing the same concept and principle used in case of modern principles of medicine. The main concept of Unani medicine is to assist in the natural recuperative power of the body and to eradicate any disease or abnormal condition from the body through the process of sweating, urination and defection. This is again in accordance to the modern ADME principles of allopathic medicine where the each of the letters stand for Absorption of the drug, Distribution of the drug, Metabolism of the drug followed by Excretion from the body. Therefore in Unani medicine before prescribing a drug to the pregnant mother the Unani practitioner needs to emphasize on the diet regime, as correct diet and digestion along with a good quality of life can help in a safe pregnancy period.

Pharmaco environmentology, another branch of Unani medicine, is important as it deals with the entry of chemicals, drugs and therapeutic molecules into the environment from the living systems post therapy [17, 28–30].

Reasons for adverse drug reactions in herbal medicines related to the Unani system of medicine can be associated with individualizing of treatment by the patients themselves, parenteral use and most importantly, adulteration [1]. The regulatory environment including the legal status of herbal drugs, their rationale and mechanistic approval for actions are also unknown as many of these approaches are based on holistic and traditional beliefs. However, generally, most of the herbal drugs used in Unani medicine are deemed to be safe as many of them are used in daily life ranging from cooking to the early stages of pregnancy till the late phases in life [17].

In Unani like in Ayurveda, therapeutics are based on a constitutional approach and the medicinal herbs are selected or excluded according to their compatibility or incompatibility to the constitutional makeup of a given individual. Unani like Ayurveda and some tribal medicines, is also dependent on the patient's constitutional health plan [31].

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42 Applications of Traditional Chinese Medicine Concepts in the Safety and Development of the Fetus During the 1st and 2nd Trimesters of Pregnancy

Priyodarshi Sengupta, Akash Bhattacharya, and Niranjan Bhattacharya

Introduction

Chinese medicine is a relatively new concept or rather an alternative medicine to the west [1]. Chinese medicine is considered to be an assimilation of philosophical and scientific thoughts and ideas that influence us in our daily lives. Chinese medicine believes in Yin Yang which metaphors the idea to a dualist reality that prevails in our society today. Like Ayurveda, Chinese medicine also represents the different elements of nature like wood, fire, metal, water and earth. Qi another concept in traditional Chinese medicine (TCM) is regarded as the main force of life filled with energy [2]. It is compared to the hot steam that comes out from cooking rice and consists of the wind, cold, heat and damp. In modern civilization Chinese medicine is slowly becoming the main stream of medicine in many east and Far East nations and is often regarded as an alternative source of treatment [3]. Since 5000 years and beyond Chinese medicine has offered many therapeutic potentials in pregnancy related indications ranging from infertility issues to threatened miscarriages [4, 5]. Chinese herbal medicine, diet and acupressure/acupuncture are some of the widely evolved and accepted Chinese method of practicing medicine. Although Chinese medicine is considered safe and gentle by some health care practitioners, public health workers and and therapists, major concerns pertaining to its safety and therapeutic potency remains a big question till date.

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Pharmacodynamic and Pharmacokinetic Principles of Chinese Medicine

The basic principles of most drugs are its physicochemical properties which govern the binding of the drug with the plasma protein. There are certain other important factors influencing the pharmacokinetics and pharmacodynamics of a drug like dosage and time and route of administration, age and any pre-existing conditions. Whether a drug should be taken as a single dose or a multiple dose and the issue of how long it should be taken

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to elicit a minimal therapeutic response, is a major factor in governing the properties of drugs.

Age is another important consideration; which drugs with what mode of action, in what type of population (whether geriatrics, paediatrics or pregnant mothers), plays a major role in deciding the drug activity and its efficiency. Any pre existing conditions like liver cirrhosis or hepatitis or renal disease like proteinuria where the proteins escape from the renal tubules, and even dermatological constraints like eczema or dermatitis, can seriously malign and impair the proper metabolic and excretory functions by which the drug can be safely metabolized and eliminated from the body.

In case of Chinese medicine, especially in case of herbal medicine, like any other principles of medicine, there is a debate whether a whole plant extract or a single plant extract is more effective and superior for treating a certain ailment. In western medicine a certain purified drug can have a certain beneficial effect on a certain disease but this comes at a cost of certain degree of side effects. Chinese herbalists believe that a whole plant contains a cocktail of different factors and compounds that not only facilitate to negate a certain disease but also help to reduce the other side effects by entering into those organs where there is a maximal risk of metabolite interference produced from the main compound. Also in a mixture or cocktail of compounds a potent chemical can act both as an inducer and inhibitor at the same time as observed in the case of the Chinese herb Dang Gui Radix Angelicae sinesis which can contract and relax the walls of the uterus at the same time and Ren Shen Radix Ginseng which can both stimulate and inhibit the CNS. Therefore the above ideas conclude that an isolated and purified content of a Chinese herbal drug may not be potent enough to elevate the same level of therapeutic potential as a whole herb would do, thereby highlighting the point that a mixture of several drugs has a much higher therapeutic activity than a single extract. As observed and stated by Borchers et al., this can be attributed due to the synergistic or antagonistic mode of the extract interaction as observed and stated by Borchers et al. [2, 6].

Also Chinese herbalists have laid down a fourfold structure to make it easier for formulating prescriptions of Chinese drugs. According to them the first type of classification is known as the Emperor herb which forms the main prescription, followed by the assisting herb known as the Minister herb. The third type is defined as the Assistant herb and its main role is to moderate the influence of the first two herbs and thereby counterbalance and negate their side effects. The fourth classification is termed as the Messenger herbs and their main function is to help in directing the drugs to a specific organ of the body [2].

Certain medications like Huang Lian, An-Tai-Yin have been found to be associated with increased risks of congenital malformations in the first trimester of fetal development such as congenital nervous system malformations including the eyes, musculoskeletal and connective tissues. However a direct causal relationship of the interactions of these drugs with the fetal malformations cannot be established yet as more scientific explanations and observations are found wanting. In the case of An-Tai-Yin it is advised to use these drugs in the third trimester in order to help in the smooth delivery process which is also similar for another drug, Huan Lian, which is used for improving skin conditions. It should be allowed from the second trimester to evade complications to the fetus [7]. Further observations from Chuang CH et al. [8] suggested that there was a non significant slight increase in the birth weight as well as an increased risk of low birth weight when Huan Lian was administered to 9,895 pregnant mothers for more than 8 weeks.

TCM and Fetal Development

Several pre eleventh century Chinese texts describe the conception and development of the foetus. Though some essential elements of these theories remain unchanged, the later works, fabricated in principle from the earlier ones reflect a progressive sophistication of concepts professed by the former, often maintaining several simultaneously. Conceptions which were initially philosophical, by the medieval period, shifted to a great extent in focus towards the medical aspects of the health of the mother and the foetus. Having been conceived in a patriarchal regime of thought, early discourses on the subject focus on the pain

of pregnancy as an intense experience rather than joy. Therein lay a fundamental social dilemma – it contrasted strongly with the (at the time) stigma of obligatorily fathering a male child [9].

The following text provides perspectives on fetal development.

Fetal development is described in the Huainanzi as follows [9, 10]

> In the first month the lard occurs
> In the second month a lump develops
> In the third month an embryo forms
> In the fourth month the flesh is produced
> In the fifth month the muscles form
> In the sixth month the bones develop
> In the seventh month the foetus forms
> In the eighth month the foetus starts to move
> In the ninth month its movements become more pronounced
> In the tenth month, the birth occurs
> In this way, the physical body is complete and the five orbs (Zang) are formed

According to Chinese medicine, the general view of pregnancy is philosophically related with respect to Qi and Yi Yang. Chinese medicine also has a way of interpreting the development of the fetus with respect to the natural elements which is denoted below.

The wood resembles the first to fifth week of fetal liver development and is related to Hun as the ethereal soul that carries messages from the forefathers.

Fire resembles the heart between the 8th and 16th weeks and is resembled in Chinese medicine as Shen, the spirit that animates us and with which we live for the rest of our lives.

The Earth resembles the fetal spleen between 16th and 24th weeks and is signified as Yi, the ability which is based on learning remembering and reflecting.

Metal represents the 24–32 weeks lungs and in Chinese medicine it is denoted by Po an earthly soul which gives humans their identities in the form of body.

Water resembles the fetal kidney during the 32th and 40th weeks of development and is classified as Zhi which gives us the will and desires to live in this world [11].

Conception is generally regarded as the first essential step towards a fruitful reproduction and according to Chinese medicine both pre natal and post natal essences combine together to form the embryo which in other words means that the maternal and paternal health at the time of conception has a profound effect on the future health and well being of the child. Hence this indicates that both the mother and the father need to have a sound health at the time of conception. After conception it is regarded that the mother produces kidney essence increasingly to provide greater nourishment to the fetus via the maternal blood circulation, as well as an increase of the functional efficiency of the different organs of the body. Qi in the meantime, depending on the regulation of the emotions, ascends for the maturation and development of the breasts in the mother and dark lines along the lower part of the abdomen. The side effects of Qi are however noted as morning sickness, mouth sores and changes in body temperature, hemorrhoids and an increase in appetite. Strong emotions can have inhibitory effects on the fetus. To help the smooth flowing process of Qi, yoga, exercises and acupressure techniques are encouraged and the mothers are constantly asked to undergo a normal stress free life with adequate and sufficient rest and maintain a healthy risk free diet [11].

Role of Diet, Exercise and Acupressure in the Initial Stages of Pregnancy

Like traditional medicines practiced in India, Ayurveda and Unani, Chinese medicine also believes that during the initial phases of pregnancy, especially in the first trimester, the body's Yin and Yang, stomach's Qi and circulation change. Which is quite similar to concepts currently prevailing in western medical science. Therefore in order to negate any inhibitory effect to the fetus, maintaining a balanced and a healthy diet is the utmost need of the day. Some traditional medicine books including the famous herbal book *Ben Cao Gang Mu shiyi* have stated that lemons are neutral in characters and primarily work on the stomach by keeping it cool and calm [12]. Lemons can be also used for treating varicose veins in pregnant mothers during lymph massage along with lavender essential oils. Morning sickness

accompanied with nausea and constipation, common side effects of pregnancy, can be treated by acupressure methods and by avoiding rich foods with ample exercise and yoga involving the stretching and massaging of the lower abdominal walls on a regular basis. Also, nausea can be treated by simply taping a magnet or a wrist band to provide constant pressure. Heart burns in the second trimester a common symptom in pregnant mothers can be reduced by taking sufficient apples and cabbages, nettle lemons, balm tea and peppermint three or four times each day. With growing gestation period as the mass and volume of the uterus increases, a constant pressure on the backbone also increases resulting in a chronic back pain. This can be treated with lavender and lemon essential oils massage. In cases of dehydration and edema along with muscle cramps, higher intake of barley, squashes accompanied with garlic acid is encouraged. Other complications like anemia, hypertension, and hemorrhoids are treated with herbal diets like passion flower, special herbal teas as an infusion for stabilizing the fetus [11].

It is advised that internal use of oils in pregnant mothers should be avoided along with different nervous system muscle stimulants like ephedra, kola nut and gurana as well as highly concentrated herbal extracts.

Diet, discussed later, in the context, is an essential and compulsory content of Chinese medicine. Foods like carrots, dark leafy and root vegetables, sprouted seeds, Brazil nuts and flax seeds are encouraged to be consumed by the mother for proper intake of protein, calcium and vitamins.

Chinese Medicine in the First and Subsequent Trimesters of Pregnancy and Dilemmas Pertaining to Its Usage

Due to the lack of standardization and characterization of the different Chinese drugs and their implications in pregnancy, utmost care by the Chinese therapists should be taken in prescribing remedies especially during the first trimester period as this is often the most sensitive period. Organogenesis initiates from this stage and it is during this period that the nervous system starts its initial development. Its primitive function is noticed between the 15th and 25th day post conception followed by the development of the eyes and cardiac tissues between the 20th and 40th day. The lower limbs also start its shaping between the 24th and 36th day of gestation period. Therefore being a very sensitive period for the fetus it is highly recommended to abstain from prescribing any Chinese medicine in the first 3 months of pregnancy; this is similar to western medicine, as any adverse events stemming from Chinese medicine can result in having serious implications on the fetal health often causing teratogenesis or mutagenesis [2].

To assess the effects of teratogenesis in pregnant mothers a comparative study was conducted at a Hong Kong University Hospital where it was found that 2.7 % of women who undertook western medicine underwent termination of pregnancy because of anxiety compared to none in case of pregnant mothers who took Chinese medicines [12]. There have been reported cases of teratogenicity related to the use of Chinese medicine during pregnancy. Takei et al. (1997) reported that a mother who took Lei Gong Teng during the initial trimester's period gave birth to a baby with occipital meningoencephalocele and cerebellar agenesis. A second example of such teratogenicity as reported by Koren et al. (1990) resulted in a baby with excessive hair, a health condition also known as androgenisation [13]. Therefore like western medicine, Chinese medicine has also been known to cause serious side effects many of which might get unreported due to lack of resources or awareness among the Chinese medical health care practitioners thereby suggesting that Chinese medicinal herbs are not always safe for pregnancy and should be handled with caution before prescribing them to pregnant mothers [14].

Also, after 3 months of pregnancy that is when the second trimester starts and organogenesis is already in a matured stage, careful considerations should be made in selecting the dosage of the drugs starting off from a low sub lethal toxicity dose and increasing it accordingly without any

observable side effects. In cases of patients with poor prognosis they can easily opt out of the treatment regimen without causing any further harm to the developing fetus [2].

Below are given the list of certain Chinese herbal medicines which are identified as having very strong Qi and blood movers and if not used appropriately can result in serious side effects during the time of pregnancy.

1. Semen persicae Tao ren
2. Flos carthami Hong hua
3. Flos carthami Hong hua
4. Rhizoma and Radix Rhei Da huang
5. Fructus aurantii Zi shi
6. Fructus aurantii Zi shi
7. Radix aconite Fu zhi
8. Rhizoma zingiberis Gan jiang
9. Cortex cinnamomi Rou gui [15].

Is Chinese Medicine a Viable Solution and an Alternative to Modern Medicine?

Different studies in both animal models and human clinical trials have failed to report serious side effects, which can be attributed to the fact either due to the lack of safety awareness among the research and clinical groups or the level of understanding regarding how these Chinese medicines act on the fetus. Here, knowledge is severely limited due to lack of definitive well controlled in vivo and clinical studies where the use of placebo and double blind trials can generally reduce the level of doubt regarding the effectiveness of a medicine bias for such findings. In a case study of WS model of Chinese herbal medicine for pregnancy as reviewed by a German research group it was found that the scientific integrity of the animal model studies were severely lacking consistency. The group as reported had a total of 1751 animals of which only 46 animals were under control trial thereby indicating that there can be large fluctuations in reporting adverse events as the group was 38 times bigger than the control models. Safety aspects of Chinese herbal medicine in pregnancy—Re-evaluation of experimental data of two animal studies and the clinical experience [1]

Also, another important issue can be the combinatorial treatment of Chinese medicinal drugs with allopathic medicines. Dissemination of safety datas from such reports may not be possible given the current stage of clinical research [16].

How Safe Are Chinese Medicinal Herbs?

Chinese medicine is not immune to adverse events after its application. However lack of animal datas, proper controlled clinical trials without scientific explanations, proper training, lack of awareness, poor follow up and personalizing Chinese medicine has resulted in under-reporting of many cases of adverse events properly [1].

According to the author of this manuscript such adverse reactions to Chinese herbal medicine could be due to either wrong identification of herbs or interactions with heavy metals thereby contaminating the drug; including another factor could be its combined use with western medicine.

Wrong diagnosis, like in the case of any medicine along with bad practice and self-medication are some of the major problems and the root cause behind the occurrence of adverse effects of Chinese drugs [2]. One such example of bad practice resulting in adverse events is reported in the use of Chinese herbs in Hong Kong causing aconite poisoning [17].

Combined treatment of Chinese medicine along with western medicine has been found to malign the many therapeutic effects of the Chinese drugs as opined by many schools of thoughts among the Chinese therapists. However, two randomized control trials involving 130 participants were analyzed for toxicity and adverse events among a group of 130 participants for combined Chinese and western medicine and no adverse reports were observed in the group. under combined medicine but On the other hand, around 2–8 % minor adverse drug effects were reported in case of Chinese medicine when applied alone [18, 19] thereby raising the question of whether Chinese drugs should be administered alone or in combination doses.

Other Alternative Treatment Strategies of Chinese Medicine during Pregnancy

Acupuncture has been used successfully in treating infertility. Pregnancy rates in Assisted Reproduction Therapy were analyzed in a prospective randomized study in 80 patients with acupuncture. Results showed a higher rate of fertility in women receiving acupuncture than the control [20]. It has been shown to demonstrate beneficial hormonal responses in threatened miscarriage, with reduced miscarriage rates [21]. It has been used to treat back pain in pregnancy. Studies show that the mechanisms of regression of symptoms of pain in acupuncture are related to the production of endorphins [22]. Acupuncture has been used for amelioration of nausea and dry retching in pregnancy. A RCT conducted in Adelaide showed a significant improvement ($p < 0.01$) in both parameters, although a placebo effect was found among some individuals [23]. Apart from these, acupuncture has also been used to treat tension type headaches in pregnancy, dyspepsia, emotional complaints and insomnia [23–27].

Other general benefits of acupuncture include improved uterine lining, increased blood flow to the uterus, hormone regulation and in alleviating stress.

Acupuncture practices are generally regarded as safe. Adverse events of acupuncture in pregnancy have been reviewed. Total AE incidence was 1.9 % and AEs evaluated as certainly, probably or possibly causally related to acupuncture was 1.3 %. All AEs were mild or moderate [27]. Most of the studies regarding the use of Chinese medicine in the 1st trimesters are based on weak observations and less follow up time period. Also it has been noted that most of the end point of the studies were related to the normal delivery of the birth without a scientific insight into fetal wellbeing during the early gestation period that might where there is a possibility that acupuncture treatment may result in give rise to silent mutations and diseases even after a healthy delivery. Therefore most of the trials designed are based on weak clinical outcomes and parameters.

On the other hand, because of fewer adverse events reported from the use of Chinese medicine in pregnant mothers, pregnant mothers may wish to use Chinese medicines during pregnancy. The traditional Chinese medicine approach to health and disease dictates that all aspects of a system must be considered when treating a problem. Only with a comprehensive analysis on the patients' physical condition, age, lifestyle, medical history, career and mental state can a correct diagnosis be made and then treated properly. Though some herbal preparations might be toxic, they are widely used because of the prevalent ideas of their safety. Some are known to be effective in various complications of pregnancy. Their effects can be potent, yet their use is not controlled. Their guided use can curb unwanted side effects. But there is no pressing need to address the efficacy of these treatments and they may be used in moderation as a supplementary to conventional treatment. Advances in the field of quality control are important for the standardisation of the practices of TCM and will allow for a better control of the bioactivity and interaction of the ingredients and in the identification of its active principles. Currently practice is not standard and opinions of practitioners vary widely. Its clinical appropriateness should be analysed by randomized controlled trials and specified for particular conditions.

Discussion

TCM and Ayurveda are the two most common types of TCAM that have been practised and prescribed not only in Asia but throughout the globe for a long time. TCM and its treatment is more common in the eastern parts of Asia. TCM is primarily focused on and is concerned about the symptoms or symptomatic manifestations whereas Ayurveda, Unani and some tribal medicines are dependent on the patient's constitutional health [28]. Also traditional Chinese medicine or TCM is focussed more on the evaluation and differentiation of syndrome [29]. In terms of pathology Chinese medicine believes more in physical examinations including the organ structure. TCM medicinal herbs are classified according to the therapeutic effects of

the herb itself, namely, dispersive quality, Yin tonifying quality and so forth. Also the Chinese Suwen and Indian Caraka of Ayurvedic system both represent the steps taken in daily life to promote one's health. It also describes different causes of diseases such as environmental, dietary and emotional factors. The main focus of Suwen is based on acupuncture. In terms of diet the Chinese system believes that normal digested food produces normal qi and blood which promotes health and well being. Chinese medicine depends on tastes like sweet, sour, salty, pungent (acrid), bitter, and bland. However during the time of pregnancy all Traditional Complementary and Alternative Medicine (TCAM) advise the mother to have a stress free life, abstain from smoking and drinking, maintain proper hygiene, consume foods that would keep the body devoid of toxins and would help in the nourishment of the fetus. Therefore all of the TCAM's lay emphasis on the standard quality of life of the pregnant mother and her happiness.

However comparing other branches of TCAM's like Unani, Ayurveda, Homeopathy and tribal medicine it can be noted that Chinese medicine has a more prominent stake in the global alternative pharmaceutical industry rather than Ayurveda or Unani. Some of the reasons can be attributed to the fact that during the past 25 years, numerous schools of Chinese medicine have opened in the west, and currently in the U.S, 36 states has licenses to formally allow the practice of acupuncturists as health professionals [30]. Acupuncture, an alternative to physiotherapy, is an exciting concept that was embraced by the west some 30 years back although now Yoga or Asanas from the Ayurvedic system of therapy are also gaining more prominence in western culture. Lastly the presence of a large Chinese diaspora has lead to huge exports of Chinese medicine in the west and has eventually helped Chinese herbs and traditional medicine to catch global attention.

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General Perspectives of Homeopathic Medicine During the First and Second Trimesters of Pregnancy

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Introduction

Homoeopathy is a system of medicine developed by Dr. Samuel Hahnemann, a German Physician who lived between 1755 and 1843 [1]. Homoeopathy is a scientific method of treatment that is based on the application of the law of similars and potentization. The law of similars states that "a substance that causes, in a healthy person. Symptoms similar to those of a disease state can cure a sick person in that similar disease state". The Latin phrase Similia Similibus Curentur that means "likes cured by likes". This law of similars is based on years of observation and a number of discoveries and reflections found throughout the history of medicine, long before Hahnemann's time. Even Hippocrates, the "father of Medicine", had stated that cure could be achieved through the action of "Similars". Hahnemann first experienced the law of similars while translating a textbook of medicine in which it was reported that Cinchona bark was used to cure malaria. He decided to test Hippocrates' theory by taking some Cinchona bark himself, and found that as a healthy person, he actually developed symptoms very similar to those of malaria. This led Hahnemann to develop a hypothesis that Cinchona appears to cure malaria because it produces the symptoms of malaria in healthy people [1, 2]. Potentization on the other hand is a process by which serial dilutions along with succession or vigorous shaking is applied to prepare homeopathic remedies. This process of alternative dilution and shaking is thought to remove the toxic side effects of the main component of the drug to have a deeper curative property of the drug.

The relative safety of Homoeopathic medicines makes them invaluable in pregnancy. "There's nothing safer", says Ananda Zaren, a nurse, midwife and Homoeopath in Santa Barbara, California, who has used Homoeopathic medicines in hundreds of births. Zaren adds,

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"The medicines help strengthen the woman physically and psychologically".

Homoeopaths differentiate the symptoms between acute symptoms and chronic symptoms. Acute symptoms represent self protective efforts of the organisms dealing with some type of recent stress or infection whereas chronic symptoms refer to recurrent, unsuccessful efforts of the organism to re-establish health. Instead of prescribing a medicine primarily for the most prominent symptoms, the homoeopath may prescribe 'Constitutional Medicine' which is individualized to the totality of a woman's symptoms [3, 4].

Jaceques Imberechts, M.D., a respected Belgian homoeopath, notes, "The Homoeopathic medicines are very effective in healing so many symptoms and syndromes of pregnancy that I have found that my patients rarely request or need anything other than Homoeopathic treatment" [5]. Richard Moskowitz, M.D., a Boston homoeopath, has found the best results in treating varicose veins with Pulsatilla and Hamamelis [6]. Marcel Simons, M.D., a Belgium obstetrician and homoeopath has also observed good results with these medicines [5, 6].

Why Homeopathy and Traditional Complementary and Alternative Medicines (TCAM) Are an Increasingly Popular Field of Medicine: Their Current Status in Different Countries

In 1991, USA instructed the National Institute of Health (NIH) to create a new federal office that will follow FDA guidelines; this came to be known as the Office of Alternative Medicine (OAM). In the UK, although chiropractice and osteopathy are officially regulated, incidence of TCAM usage is around 40 %. In other developed countries usage of TCAM is comparably low, for instance, it is 46 % in Australia, 70 % in Canada, 31 % in Belgium and 49 % France However, in the developing countries, the usage of TCAM is quite staggeringly high with Ethiopia reporting the highest usage of these forms of drugs at 90 %, followed by Benin at 80 %, India and Rwanda at 70 % while Tanzania and Uganda are at 60 % [7].

This might be attributed to the fact that in developing countries, cost is a major factor as many people cannot afford allopathic drugs due to socio-economic and financial constraints; therefore TCAM ha become a better option for treatment. Another important area which is the emergence of more and more chronic diseases in the developing countries [8]. Harmful side effects of allopathy can be one of the main reasons why the use of TCAM is gaining grounds among many patients in the west and developed nations [8]. A survey carried out in Chhattisgarh, a state in India, reported that 41 % of people were satisfied with ayurvedic drugs [3]. However a negative trend was observed when around 94 % of 492 respondents in another study, preferred allopathic drugs, with 3.25 % favouring ayurvedic and 2.85 % homeopathic drugs, in spite of another opinion expressed by the same respondents that the biggest side effect was associated with allopathic drug at 93.9 %, followed by 3.45 % in ayurveda, and 2.65 % in homeopathy [9]. Also it was found that ayurveda was the preferred choice of medication in case of long term treatment followed by allopathy and homeopathy whereas people found allopathic medicine to be the most common medicine that the group would opt for in the first instance [9]. Therefore the above data reveals that ayurvedic and homeopathic medicines perhaps play a prominent, maybe a dominant role, among patients when it comes to opting for the best suitable treatment.

Rationale behind the Usage of Homeopathic Medicine: a Perspective into Some Clinical Trials

Before moving into the perspectives and usage of homeopathic drugs during the first and second trimester of pregnancy and their role in growth and development of the fetus it is important to consider certain facets of homeopathy in terms of scientific principles. One of the major challenges of homeopathy like other traditional complementary and alternative medicine is its lack of definitive clinical data in terms of clinical trials which can be attributed to the fact that most of the trials lack a sound clinical design and approach [10]. A study of osteoarthritis in a group of 36 patients concluded that one third of the patients who received a dose of 30× Rhus Tox showed no bet-

ter symptoms than the other placebo group, whereas a third group showed some response to standard care of NSAID's. This team, when they conducted a similar study on hay fever symptoms with a 30× dose of pollen grains processed, found that the group which received the homeopathic treatment showed six times fewer symptoms than the placebo group. Also against radiation in albino rat, homeopathy has shown less chromosomal aberration and cell death with respect to placebo albino rat. Also another study in an animal model reported that homeopathy can reduce the reddish appearance and infer a protective role against causing inflammation of the skin when exposed to X-rays for long periods [11].

Homeopathy has now become a major contribution of alternative and complementary medicine to many countries in Europe. In the UK 17 % of people use homeopathy according to a House of Lords report in 2000 [10]. Also another study found that 55 % of English people related homeopathy to be a useful medicine against a mere 14 %. In Ireland a 4 month study conducted in children found that 57 % of parents reported using CAM [12] for their children among which usage of homeopathy were 16 % which was also incidentally the highest and the most common of these CAM medicines used among the respondent groups [12]. According to a 2004 study, statistics showed that in France, 62 % of French mothers used homeopathy 12 months ahead of their pregnancy [13]. Also another study found that around 95 % of French pharmacists recommended prescribing homeopathic medicine to pregnant mothers [14]. In Germany during 1993, 1993 trained homeopathic physicians were present which rose in 2006 to around 6,073 trained physicians in this branch of medicine [15].

Fetal Development

Events during the First Week of Human Development

1. Oocyte immediately after ovulation.
2. Fertilization, approximately 12–24 h after ovulation
3. Stage of the male and female pro nuclei.
4. Spindle of the first mitotic division.
5. Two cell stage approximately 30 h of age.
6. Morula containing 12–16 blastomeres approximately 3 days of age.
7. Advanced Morula stage, reaching the uterine lumen at approximately 4 days of age.
8. Early Blastosyst stage, approximately 4.5 days of age, the zona pelucida has disappeared.

After 9 or 10 days of conception the blastocyst is fully attached to the endometrium. Primitive placental blood circulation begins in 12 days or so after conception. In the meantime the blastocyst starts to produce hormones like Human Chorionic Gonadotrophin Hormone (hCG) which is essentially one of the most important hormones responsible for inferring selective safe passage across the transplacental membrane (Miasm-Psora). This hormone is also detected in women's urine defining the start of pregnancy. It is in this stage that the fetus starts its development.

Stages of Development of Fetus after Fertilization

In the first stage of development, the embryo is called a zygote and with subsequent divisions termed as cleavage the different stages of development take place like the formation of morula, blastomere compaction resulting in the formation of blastocyst with the blastocoel cavity. The blastocyst contains the inner cell mass, a rich and potent source of embryonic and pluripotent stem cells. Appearance of epiblast, hypoblast, extra embryonic mesoderm and visceral mesoderm along with the trophoblast is initiated.

First Trimester

Following 13 or 14 days after conception, a "primitive streak" appears which later develops into the fetal central nervous system. The preembryo is now referred to as an embryo. Structurally this embryo appears to be a very small blob of undifferentiated tissue at this stage of development. In 3 weeks time the embryo

matures. The cardiac muscle slowly develops and in about 3 weeks time light heart beat can be observed. In the third week after conception the in utero size of the fetus matures to 1/5″ long and resembles a tadpole. The head, which starts developing in this organogenesis phase starts developing and to compensate fetal breathing activities, structures resembling like those of the fish gills develop in the future throat area. Tiny arms and leg buds start forming from 5 weeks time. Hands with webs between the fingers are formed at the end of the arm buds. The face has a distinctly reptilian aspect [16–18].

In 6 weeks time the embryo is about 1/2″ long. The face has one eye on each side of its head. The front of the face has connected slits where the month and nose eventually develop. By the time of the seventh week the embryo almost loses its tail. The face is mammalian but is somewhat resembles pig-like. Pain sensors appear. However the higher functions of the brain are yet to develop and the pathways to transfer pain signals from the pain sensors to the brain are still immature. In 2 months time the embryo's face resembles that of a primate although not fully human in appearance. Some of the brain regions begins to evolve and mature; this is the primitive "reptilian brain" that functions throughout life.

At 10 weeks time the embryo is now called a fetus. Its face looks human and its gender may be detectable via ultrasound. The fetus is about 3 in. long by the time it reaches 13 weeks time and weighs about an ounce. Fingernails and bones can be seen [19].

Second Trimester

During second trimester, i.e., after 17 weeks or 3.9 months of conception the fetus is about 8 in and weighs half a pound. The movements of the fetus may begin to be felt. Its heartbeat can usually be detected in the mother's abdomen. In about 22 weeks or 5 months the size of the fetus further increases to anout 12 in. in length and it weighs about a pound and fetal hair starts to develop on the dermal side. Its movements can be felt [20, 21].

In about 26 weeks or 6 months the fetus is 14 in. long and almost 2 lb. In the meantime the lung bronchioles develop. Functional interlinking of the neurons initiates the higher functional development of the brain for the first time. This is the time that the fetus is able to feel pain for the first time [6].

Management of Homeopathic Medicines in First and Second Trimesters of Pregnancy

First Trimester

Morning sickness is one of the first signs of pregnancy. It is often characterized by a feeling of nausea or sickness and in many cases it can induce vomiting too. This symptom usually lasts until the patient is 14–16 weeks pregnant but can occasionally persist through the whole pregnancy. If very severe, it is referred to as "Hyperemesis Gravidarum" and may require a hospital admission and/or conventional medication [17, 18, 22]. This can be treated by Nux Vomica if the vomiting is worse in the morning. Coculus Indica derived from the cockle flower can be very helpful if nausea starts immediately after lifting the head from the pillow [23].

Due to the changes in hormonal regulation breast tenderness is another common ailment that is often observed among pregnant mothers in the first trimester. Pulsatilla can be recommended as a remedy usually taken 30 c once or twice daily as it normally helps to soothe the tenderness [23]. Also, Natrummur, Belladona and Phytolacca may be suggested [3, 24, 25].

Treatment

Sepia is one of the most important remedies during this pregnancy period where the smell of food triggers sickness and eating does not help as it aggravates the problem of vomiting. Different drugs used for treating this symptom are Arsenicum, Ipecac, Kreosote, Symphoricarpus Racemosa, Cuprum ars, Gossypium, Iris V and Carbo Veg [5, 16, 26, 27]. Urinary frequency in

the mother is increased which can be due to the pressure of the gravid uterus during first trimester. Treatment includes Causticum, Coccus Cacti and Merc. Sol which can be used to treat sour smelling urine.

Second Trimester

Backache is a common ailment during 3–6 months of pregnancy as the bump will be getting bigger creating pressure on the spinal cord region including slight and tender inflammation of the pelvis enlarging as the ligaments stretch due to the effect of pregnancy hormones.

Treatments in homeopathy include drugs like Kali. Carb, Natrummur and Arnica.

Constipation, another common syndrome in pregnant mothers, is due to added pressure on the bowels from the growing baby and also due to the effects of the hormone progesterone.

Treatment includes Lycopodium, Alumina, Nux vomica, Opium and Platina.

Haemorrhoids like constipation can be treated by Collinsonia, Lachesis and Sulphur. Varicose veins are treated by apismel, Arnica, Carbo Veg and Hamamelis [28–30].

Due to the maturation of the fetus in the second trimester the womb of the mother considerably increases in size thereby putting pressure on the pelvic main arteries and the veins. This results in the feeling of faintness while lying back. This phenomenon is known as varicose veins. Avoiding long periods of standing during this period is normally recommended. However the latter is not possible in case of working women. Pulsatilla of dose 6 c twice daily can be prescribed. Carbo Vegetabilis can be recommended in cases where Pulsatilla is not proving to be effective.

Anemia in Pregnancy

Anemia is common in pregnancy and it has been described as the second leading cause of maternal death. According to WHO guidelines, anemia during pregnancy is considered when the hemoglobin level <11.0 g in pregnancy, but as per the Indian standard it is considered when hemoglobin level is less than 10.5 g. As the pregnancy progresses, the blood becomes diluted and the woman may become anemic. The dilution of blood in pregnancy is a natural process and starts at approximately the 8th week of pregnancy and progresses until the 32nd–34th week of pregnancy.

Requirement of Iron: 0.8 mg daily in the first trimester and 4–5 mg daily in the second trimester [29].

Treatment

Homeopathy offers a number of remedies that may be helpful in treating anemia. Homeopathy considers anemia as the symptomatic results of some other underlined conditions and attempts to treat these conditions. Before prescribing a remedy, homeopaths should take into account a person's constitutional types and then accordingly determine the most appropriate treatment for each individual. Indications of some of the commonly used Homeopathic medicines are as follows [20, 21].

- Ferrum Met.
- Ferrum Phos.
- Pulsatilla
- Cinchona
- Nat. Mur
- Acetic acid
- Calc carb
- Calc Phos
- Alumina
- Nux Vomica
- Arsenicum
- Kali Carb

Concepts of Homeopathy with Respect to Modern Medicine

With respect to modern medicine many of the theories, ideas and logic behind the use of TCAM's are scientific although no scientific answers exist. For example, homeopathy believes

that there are diseases that are already manifest in the human body, which is quite similar to the modern medical concepts, for instance, aberrations of cancer controlling genes or activation of the gut flora that can cause serious implications to our health [31]. On the other hand most homeopathic drugs are manufactured on the basis of dilution principle which raises the question as to how can diluted drugs elicit potent activity inside the body if they do not have a minimal therapeutic threshold, or rather, what are the criteria for designing a homeopathic drug with a minimal response level to negate the side effects of the drug constituents. According to homeopathic physicians, to maintain the safety of such drugs the potency of the main drug composition is reduced into serial dilutions [31]. This concept of reducing the harmful components of a drug by diluting it is sometimes very confusing, showing that due to lack in scientific design of clinical trials and experiments, most homeopathic manufacturers or clinicians remain confused [31]. Such occasional anomalies question the very scientific basis of treatment of diseases by Traditional Complementary and Alternative Medicines (TCAM) and raise serious questions regarding their validation and importance [31].

Therefore homeopathy focuses more on the curative properties of diseases which are already manifest in the human body by means of diluted drugs which again is a concept under the scanner by many theorists and scientists [31].

Conclusion

Homoeopathy is a very specialized therapy. Remedies are prescribed on the basis of a person's individual symptoms. The medicines are very mild and given in minute doses, hence they are perfectly safe to take during pregnancy. In fact homoeopathic medicines boost up immunity of the patient, thereby allowing the patient to acquire resistance against diseases. However like every other branch of medicine more scientific and clinical data are required before the safety and efficacy of homeopathic medicines during pregnancy are clinically proven.

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44 Ayurvedic Perspective of Pregnancy and Fetal Development During the First and Second Trimester

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Introduction

Healing is defined as the concept or rather an art of the flow of energy from one part of the body to the other where it is most needed in order to re-establish a balance in the individual. This is the most basic concept on which the major traditional, complementary and alternative system of medicine has been relying upon since age old time [1]. Traditional complementary and alternative medicine also termed as TCAM encompasses a wide spectrum of healing resources such as health systems, holistic approaches, theories, beliefs and their age old practices since time immemorial. TCAM deals with that branch of medicine that has not yet been readily integrated into the standardized general health care models due to its challenges in scientific findings as perceived by the medical fraternity [1]. The word complementary or alternative medicine evolved gradually over the years when people started to use these type of medicines when they found limitations in the current allopathic system [1]. TCAM's are based on the common concept of spirituality and controlling the quality and standard of life. According to Dr. Pal, TCAM can be further classified into seven categories [1]. Mind and body medicine which deals with behavioural, psychological, social and spiritual healing is the first category. An alternative medical approach which is the next one deals with yoga, puncture, hypnosis and meditation and Qi Gong [1]. The third type is related to the application of ayurvedic principles like panchkarma, health and lifestyle promotion and electrodermal diagnosis and is termed as

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lifestyle based disease prevention [1]. The different formulations of herbal drugs and medicines used extensively and widely in case of Unani, Ayurveda, tribal medicine, Homeopathy and Ayurveda is referred to as biologically based therapies which is the 4th type. Chiropractice, massage, kinesiology, Chinese tuina massage are all related to the fifth concept of manipulative and body based systemic therapies. Biofield and bioelectromagnetic therapies which comprises of the Reiki, external Qi Gong, use of electronic magnetic fields for medical diagnosis forms the sixth and seventh basis of TCAM based medical therapies [1].

Growth and Development of Human Fetus as per Ayurveda

Ayurveda, literally meaning the science of life (ayur-life,veda = science) is an ancient medical science developed in India. It is believed to have been passed on to humans by the Gods themselves [2]. Modern conceptions of Ayurveda have developed and evolved over thousands of years, originating from the confluence of ideas of several ancient treatises. Widely regarded as the oldest form of healthcare in the world, the fundamentals of Ayurveda can be found in Hindu scriptures called the Vedas – the ancient Indian books of wisdom. The Rig Veda, written over 6000 years ago, contains a series of prescriptions that aid humans in overcoming various ailments [3]. The aims of this system of medicine are clearly delineated.

They can be summed up as follows: Swasthyas swasthya rakshanam: To protect health and prolong life; Aturasya vikar prashamanamcha: To eliminate diseases and dysfunctions of the body [4]. Ayurvedic practices mainly depend on the treatment of the three dosas (evils) of an individual, i.e., vata, pitta, kapha [5] by the use of herbal medicines, minerals or metal supplementation (rasa sastra), surgical techniques, opium and application of oils and massages in panchkarma. The practice of Ayurveda is not directed at symptomatic treatment, but aimed at the root of disease by addressing the state of imbalance of the three dosas. Methodologically, this involves their expulsion from the system by Panchkarma therapies [6]: Vaman (emesis), Virechan (Puragation) and Vasti (enema), Shirovasti(retention of medicated oils over the scalp) Nashya. (Insufflation of medicinal herbs) In Ayurveda, fetal development is described comprehensively. Health problems pertaining to the mother and the growing fetus as well as dietary recommendations finds a prominent place in a number of Ayurvedic texts.

Embryology

The union of sukra (sperm) artava (ovum) and atma or jiva inside the uterus, i.e., kuksi is known as garbha (embryo or fetus). Besides atma the association of prakriti and vikaras are also essential for the development of arms, legs, tongue, nose, ears, hips and other body parts and it is termed as sarira. As per ayurvedic classics the body is composed of five tatwas (elements), five indiriyas (sense organs) and seven dhatus (liquids, blood, muscle, fat, bone, marrow, semen). The garbha (fetus) formation is mainly due to vayu function as per Carak and Susruta; this indicates that vayu which moves agni for the formation of the five srotas (channels) therefore is the most important [7]. The garbha has six parts: (a) JIVA/ATAMA (conciousness) – This is the root element of garbha. Nothing can happen in life without atma. (b) Five mahabhutas (Elements) – Prithvi (Earth), Akash (space), Vayu (Air), Agni (Fire), Jal (Water) (C) The essential part of the garbha is ojas or sometimes called first dhatu [7] (Table 44.1).

Although Ayurvedic medicine is one of the oldest branches of medicine, many of its concepts in development of the fetus are extremely scientific, some of which may have laid the basis of modern medicine. In Ayurveda, space, air, fire, water and earth are described as panchmahabhutas and each one of these elements give qualities of life to the fetus. Looking at the development of the embryo with respect to Table 44.1, it can be observed that any deficiency in the qualities is directly linked to the five elements also known as the Panch Bhutas [9]. They are listed as below:

(a) **Vayu or air** grants the dosha body type to the fetus. It is this element that decides whether the child will have Kapha dosha type, Pitta dosha type or Vata dosha type body. Amount of ‘vayu’ in one’s body or the capability of

Table 44.1 Qualities of panchamahabhutas in the development of the fetus

Panch Bhūtas	Ākāśa (Space)	Vāyu (Air)	Agni (Fire)	Jala (Water)	Prithvī(Earth)
Qualities	Sound	Touch	Sight	Taste	Smell
	Hearing	Feeling	Seeing	Tasting	Evacuation
	Lightness	Roughness	Warmth	Coldness	Heaviness
	Smallness	Impulsion	Metabolism	Unctuousness	Stability
	Division	Activity	Enzymes	Moisture	Hair
	Srotas(channels)	Formation of dhatus Transportation of Dhatus	Luster	Blood	Bones
	Orifices	Expiration	Body temperature	Semen	
				Liquid	
				Fat	

retaining or releasing it from the body decides the dosha body type of any human being [8].

(b) **Agni or fire** decides what will be the metabolism of the baby's body. Metabolism is defined by digestive capability of the body – if agni is high, the baby will have good digestion power and vice versa. It also decides about body temperature, light and lusture, sharpness, intellect, complexion [8]

(c) **Jal or water** is called the lifeline by Ayurveda. It is responsible for cleansing the entire bodily organs. It decides about the creation of rasa or taste, coldness, softness, moistening ability of the body, blood and semen characteristics, as also of fat, muscle mass and saliva [8].

(d) **Prithvi or earth** is solid by nature. It decides on how strong the baby will be- physically as well as emotionally. It decides such structural attributes of the body as heaviness, stability, bones, nails, courage, skin, muscles, body hair etc. It also decides upon the stability and smelling ability of the baby [8].

(e) **Akash or sky** is eternal and it represents growth. It is responsible for such attributes of the baby as lightness, observing power, psych, intelligence, pride as also for lethargy, hearing ability, sleep etc**.** [8]

So, when an element is in excess or in lesser quantity in the fetus, they affect them in different ways and grant them the body as well as mental and emotional characteristics they have.

According to Ayurvedic classics it is believed that all soft parts of the body come from the mother and the hard parts of the body from the father [9].

All the classic authors have detailed accounts of the embryology and monthly development of the fetus. However a detailed description of such is outside the scope of this review. Aspects which are relevant are the desires, longing and cravings of the pregnant mother which must be fulfilled as they are indirectly linked to the desires of the fetus inside the mother's womb irrespective of whether the fetus is healthy or unhealthy [10]. It is also suggested that in order to keep the woman satisfied; both her wholesome and unwholesome desires should be fulfilled. Unfulfilled desires of the mother lead to aggravated Vata, which can either destroy or deform the fetus. On the other hand a pregnant woman, whose desires are fulfilled, will "beget a son endowed with valour and long life". The key events of human development including the fetal period following fertilization and organogenesis are quite well established clinically in Ayurvedic system of medicine. It is described in the following section.

Monthly Development of the Human Fetus According to Concept of Ayurvedic Medicine, Garbha (Embryo) Up to Second Trimester

According to *Charak Samhita* and *Sushrut Samhita*, although there are some conceptual differences between Ayurvedic and Western medicine still both these branches of medical sciences complement each other in terms of basic principles and overall similarities.

First month:

Sushrut and *Charak Samhita*: The kalal is formed in the first month which is composed of mucous similar to that of the nose and is semi solid, sticky and slimy. The kalal possesses all organs, systems and building blocks of development. It is described as the ultramicro version of a life that gets manifested later with increase in gestation period. Like each and every entity in the universe, it is made up of the five great elements namely Space, Air, Fire, Water and the Earth all combined together in different proportions [11–14].

Second month:

The embryo becomes solid in the presence of the heat produced by the kapha, pitta and vata which are regarded as the three basic energy sources of the body. The Garbha acquires either circular (pinda) or elongated (peshi) or semispherical (arbuda) form. If it is pinda, the Garbha develops into male and if peshi it develops into female. If the garbha is semispherical then it will possess both male and female characteristics [11, 12, 15, 16].

Third month:

Five pedestals/min. form (pindaka) for hands, feet, head and miniature forms of all body parts develop thus laying the very concept of organogenesis as per western medicine is concerned [17, 18].

Fourth month:

The minute forms of all body parts take specific form and shape. As the fetal heart becomes perceivable, the chetna dhatu (soul) becomes evident. This organ becomes the home of the soul. Garbha responds to sensory stimuli and that is why pregnant women have cravings (Douhrida). The woman is called Douhridini (with two hearts) [11, 12, 16].

Fifth month:

During this period according to Ayurveda the mind wakes up or becomes alert and the blood and the muscle tissue of the fetus enhances and matures. Most of the nutrition is provided to the muscle tissues and the fetal blood for its enrichment and nourishment. As the exchange of nutrients from mother to fetus enhances the mother feels weak and may start losing weight also [15, 16].

Sixth month:

The Buddhi (intellect with the power of grasping and retaining) matures or progresses mainly in this stage. The fetus continues to become strong and healthy [16].

Trends in Ayurvedic Medicine

The Ayurvedic approach towards motherhood including early stage pregnancy till childbirth is indeed a holistic one. Ayurveda through its different spiritual and philosophical ways prepare the woman for pregnancy. Due to acute shortage of trained and qualified Ayurvedic practitioners there is always a high chance of drug abuse. Also the reliability, by which Ayurvedic medicines are controlled, commercialized, marketed and regulated remains questionable. Currently there are no definitive protocols or laws in place as specified by the Drugs and Cosmetics Acts of India in order to ensure the safe marketing and commercialization of Ayurvedic medicines especially related to vulnerable groups like pregnant mothers. There are medications to control hormonal imbalances but most of the Ayurvedic medicines have heavy regulations on them. Also due to lack of proper knowledge among practitioners and general awareness among the masses one can see hundreds of quack and self proclaimed ayurved clinicians, second-hand forcible prints and advertisements which often malign the very basic principles and fundamentals of Ayurvedic treatment which is to heal with the laws of science and nature implemented together. The concept of Ayurveda is originally of herbarium medication even though Gynecology in modern medicine has many applications with natural ingredients and formulations. Some of the best Ayurvedic products of interest are Ovarin and Vatvidhwansan Ras [19–21] containing more than 24,000 ppm lead, 70,000 ppm Mercury and 1000 ppm Arsenic.

Role of Ayurveda for the Pregnant Mother and the Fetus in the Early Stages of Pregnancy

In Ayurveda the main concept during pregnancy is the science of well being of both the mother and the fetus. The mother should always try to be in a happy mood and should follow proper sanitation and hygiene like keeping neat and clean, well dressed, wear simple clothes and sleep under a roof always in a serene environment. The food she eats should be moist, tasty, enriched with nutrients mainly in liquid form. It should be also ensured that the food is enriched with all the six rasas (tastes) and treated by deepan drugs which are known to increase appetite and digestive power [20, 21]. An important and essential Ayurvedic recommendation for a pregnant mother is to control her dietary intake by avoiding rich foods and consuming more natural foods [22, 23]. Also the mother's quality and standard of life has a profound effect on her well being and behavior. Apart from ayurvedic herbal remedies and applications it is also postulated that yoga and meditation just like acupuncture in Chinese medicine can help a woman to obtain serenity and a sense of tranquility during her pregnancy period which in turn can aid in the nourishment and development of the mental health of the baby [13, 24].

Quality of life is another important aspect and concept of Ayurvedic medicine for pregnant women. Mothers during this stage should always abstain from excessive sex particularly during early and late pregnancy including overeating or fasting, sleeping during the daytime and staying up late at night. She should also avoid wearing tight clothes and tight belts, witnessing or listening to things which might give rise to feelings of sorrow, anger, horror pain or agony. She needs to avoid bumpy road rides and should restrict herself from excessive travelling and squatting for a long time or sitting in an uncomfortable position or on a hard surface. Mothers should also be warned against lifting heavy things or remaining in a bending position for a long time and oleation massage. Unless positively indicated, beholding natural urges are essential except in emergency cases [25]. Dry, fermented, heavy, hot or strong food, alcohol and meat (although fish is allowed), visiting abandoned and remote places, leaning into a deep well or change in posture that may increase the chance of harm to the abdominal position during pregnancy should be avoided [20, 21]. The general rule is to take greater care during the first 3 months of pregnancy up to the completion of the seventh month. During the first trimester, stress is laid on stabilizing the pregnancy and nurturing the uterine bed through rasa and rakta dhatus. The embryo gets nourishment directly by percolation (upsnehan) hence more jaleeya (liquid) substances such as juicy fruits, coconut water, milk and so on are advised. In the first month, sipping cold milk and maintaining light diet and during the next 2 months, the intake of milk medicated with herbs like vidhari, shatavari, yasthimadhu, brahmin which are jeevaniya (life building) and garbhasthapak (helping nidation) are encouraged [21]. Honey and ghee are also recommended during this period. By the end of the third month, the body parts of the fetus becomes differentiated, sensory perceptions and motor reactions start developing, the heart starts beating and is said to express its desires through the maternal circulation. This is the period when the woman craves for certain foods/flavors required for both the mother and fetus. Hence Ayurveda recommends that her cravings be fulfilled as per as the requirements otherwise it can lead to contraindications. Brahmi helps in calming the nerves and is also a good prajasthapan (sustainer of pregnancy) [11–14, 21].

During the fourth month and extending up to the seventh month, drugs which give strength to the uterine muscles and nourishment to the embryo are advised, e.g., ashwagandha, kraunch beej and guduchi helps to prevent intrauterine growth retardation (IUGR). Nourishment starts through the umbilical cord by the kedar kulya method. During this time the diet should be consisting of rice, milk, butter and ghee. Fruits which are orange or yellow in color such as mangoes, apples, carrots, amalaki etc. along with leafy vegetables are also advised [26]. During the seventh month, the abdominal

skin gets stretched giving rise to itching and striations which are called kikkis. It should be either treated by taking sips of the infusion of berries or butter medicated with manjistha or by the application of the pulp of sandalwood and lotus or by a paste made of neem, basil and manjistha or oil medicated with karveer leaves or jasmine.

As soon as a pregnant woman enters the ninth month, she is supposed to move to the delivery stage. After an asthapan basti (simple enema), she should undergo anuvasan basti (retention enema of oil boiled with some herbs) which may be repeated. Tampons soaked in the same oil are kept in the vagina to make the pelvis soft and elastic. It enhances excretory function of apan vayu (urination and defecation) and expulsion of the fetus. The maternal skin and nails become soft, her strength and complexion are rejuvenated. Spotting of blood during any month of pregnancy should be taken seriously by the ayurvedic clinician [11, 19–21].

Ayurveda and Modern Science in Lieu of Fetal Growth and Development

In the first month, sperm and ovum unite and they turn into seed which further divides to take a more spherical seed like structure. These further divisions are referred to as cleavage in western medicine, help in forming the inner and outer seed layers and in between them a cavity is formed which remains filled with fluids known as blastocoele in modern medicine [11, 12]. According to modern science once the zygote has reached a two cell stage, it undergoes a series of divisions, forming blastula, morula stage then blastocoele or blastocyst stage [27]. In the third month all germinating parts appear. The head is quite large and fingers become visible. Male and female sex organs are formed although the eyelids and lip are not separated still [11, 12]. Modern science states that the head and upper limbs are still disproportionately large in the 3rd month and by the 12th week, external genitalia develop which can be diagnosed by ultrasonography. During the third month eyelids meet and fuse [27, 28]. According to *Charak Samhita*, after birth, a hole in the middle flap of the heart chamber closes referred to as the foramen ovale in modern medicine concept which closes after birth [29, 30]. By the third month, the heart appears and starts pumping. Designation of the heart chambers occurs and the fetal heart sounds can be heard in the maternal abdomen around the fourth month. This also has been elaborated in a similar manner in modern science. Mucous membrane of the vagina gives a bluish tinge and this sign appears by the beginning of the second month as per *Charak Samhita* [11, 12]. In modern science, it is referred to as Jacquemiers or chadwicks sign [31, 32].

In the fourth month fetal movements are felt and fine hair like structures can be seen on head and other sites of the body. Such hair like appearances are also known as lanugo [6]. Prior to 6 months timeframe, fetal skin remains wrinkled according to Ayurveda which is again consistent with respect to modern science [18, 33]. According to Ayurveda, testes descend into the scrotum at 9 months post conception. The fetus measures 20 in. in length and 3.5 kg in weight (normal and healthy) which is in accordance to both Ayurvedic and modern medical science concept. The size of the finger nails also increase with increased gestational time which is about 50 cm in case of modern medicine [34, 35]. Therefore from the above reviews and findings it can be noted that both the concepts of Ayurveda and modern medical science are based on the same principles and concepts of fetal growth and development as stated previously in this chapter [13, 14, 19, 33, 35].

Role of Yoga in Ayurveda Therapy of the Mother during the First and Second Trimester of Pregnancy

Yogas in the First Trimester of Pregnancy

Like acupuncture, acupressure and in traditional Chinese medicine, yoga in ayurvedic medicine during pregnancy in the first and second trimester

plays an important role in helping the pregnant mother to stay active and energetic during these periods of fetal development [36].

Some of the most practised first trimester asanas are Ardha Titali asana or half butterfly as it is known, which is an excellent yoga that can help in loosening of the hip and knee joints thus enabling the mother to have a safer and a faster natural delivery. Poorna Titali asana or full butterfly plays an important role in relieving pain and tiredness from the legs. Supta Udarakarshan asana or sleeping abdominal stretch pose relieves stiffness from the spine and overcomes the problems of constipation. Tadasana and Utthanasana are also good for spinal loosening, clearing any nerve congestion, strengthening the muscles of the uterus, thighs and ankles. Kati Chakrasana is good for toning the waist, hip and back muscles whereas Kashta Takshana asana loosens the pelvic muscles. Chakki Chalan asana is good for regulating the menstrual cycle and toning of the nerves, pelvis and abdominal region. Marjariasana is known to induce flexibility of the neck, shoulders and spine area apart from helping in toning the female reproductive system [36].

Yogas in the Second Trimester

Some of the most practiced yogas or the asanas as it is known in Ayurveda system of therapy during the second trimester are listed below. Matsya Kridasana which helps in re-distributing the excess weight around the waistline helps in digestion, easing constipation and relaxing the nerves of the legs. It also helps in relaxing and regulating the sleeping patterns of the pregnant mother, who remains stress free during the second trimester. Vajrasana and Bhadrasana are good for digestion and relief from acidity. Meru Akarshanasan is good for the thigh and abdominal muscles and their strengthening whereas Marjarisana improves the neck, spine and shoulder muscle flexibility. Hasta Utthanasan or hand raising pose helps in reducing muscle spasms of the neck, shoulders and upper back and is extremely good for cardiac and blood circulation and helps in more oxygen supply to the brain. However certain asanas or yogas like forward/backward bending, Sarvangasana, Matsyasana, Sasankasana, Vakrasana and Ardhamatsyendrasana should be avoided by the pregnant mother during the time of pregnancy [36].

It is also documented that different massages with ayurvedic oils like head, breast, foot and body massages can help in relieving back pressure, pains, reducing stretch mark and can improve the overall quality of life during pregnancy [37].

Safety of Ayurvedic Medicine: Do We Have Enough Conclusive Evidence?

Six cases of lead poisoning was reported by the New York City Department during the year 2011–2012 among six cases of foreign born pregnant women with the use of ten oral ayurvedic drugs made in India. The amount of lead in the blood levels of these six pregnant mothers ranged from 16 to 64 μg/dL and also after investigation it was found that most of the medications had a lead concentration of 2.4 % as well as toxic levels of mercury and arsenic which could have caused an adverse health event [38]. Anesthesiologists (ASA) have taken a conservative stance and recommended that it is essential to stop using herbal or Ayurvedic medicine at 2–3 weeks prior to anesthesia and surgery [39, 40]. Also, an American study published that in 2004, 70 Ayurvedic medicines purchased over the counter which were all manufactured in Asia contained lead, mercury and arsenic at an alarming level. In 2004, CDC reported 12 reports of lead poisoning due to herbal medicines.

List of Ayurvedic Herbs That Can Be Used during the First Two Trimesters of Fetal Growth and Development

To reduce vomiting and morning sickness among pregnant women in the first trimester Vilvaadhi laham can be prescribed at a dose of 1–2 g for

five to six times till it dissolves in the mouth. Also drinking a lot of natural fluids like coconut water is extremely beneficial especially when the mother is experiencing stress and tiredness resulting from continuous vomiting and nausea. Also Garbha raksheena guliga or maha-dhanwanthira can be prescribed to the pregnant mother with jeera water (cumin seed) in the morning and evening once. In the second trimester 15 ml of Garbharaksha kashayam boiled in 60 ml of water and then cooled can be prescribed to the pregnant mother in the second trimester of pregnancy. Also one garbharaksheena-guliga can be added and this whole mixture can be taken 30 min before breakfast and if needed then in the evening. Also two spoons of 10 ml of dhadhi maathi hritham before lunch is recommended. After bathing everyday application of dhanwanthiram kuzhambu or thailam is beneficial to the pregnant mother during this trimester [41].

Garlic, an important herb used for cooking purposes across the world helps in boosting the immune system and reducing the chances of ischemic induced neuronal injuries [42]. It is regarded safe by USFDA and has less toxicities as observed in post operative cases of bleeding especially in case of urethral resection of the prostate gland.

Aswaganda is a sympathomimetic drug used by athletes to boost their energy levels. Adverse events like hypertension, tachycardia, hypoglycemia, insomnia, nervousness and skin rashes are observed. Turmeric or haldi works as an anti septic, anti-inflammatory and antibacterial substance, but can cause very rare iron deficiency. Tulsi is used mainly for treating respiratory syndromes, cough and cold, flu, diarrhea, malaria and cardiac diseases. Currently no known side effects with tulsi are reported as of yet. Amla is a rich source of Vitamin C which is 30 times the amount found in oranges. It helps in building up the immunity of the body, acts as an anti oxidant and free radical scavenger and preventing age related disorders [43]. Also no side effects are reported with Amla yet. Guggul helps in the treatment of heat diseases as well as a good anti oxidant, anti-obesity and hypolipidemic drug [44]. It can also help in reducing C-reactive proteins in the body thus reducing fat. Giloe acts as a cardiotonic, expectorant, analgesics, anti pyretic, anti diabetic and anti inflammatory drug [44, 45]. According to WHO 75 % of the world's population use herbal remedies for basic health care.

Pharmacovigilance of Ayurvedic Drugs, a General Perspective

The word drug has come from the ancient word "Root" and by definition herbs are drugs [39]. Herbal tea is not a problem but some traditional medicinal herbs are the problem.

Classical Ayurveda classifies metals and minerals as bhasmas which can be given in combination with plants as herbomineral formulations. Manufacturing protocols for these type of medicines are extremely stringent and can only cause adverse event if in case they are not properly manufactured [47, 48].

Ayurved in India is regarded as a formal medicine along with allopathy. It is an old system of medicine that was incorporated into the Indian medical education system over a century ago. Now the country has 196 undergraduate colleges and 55 postgraduate centres where Ayurveda is taught. Currently 438,721 [46] licensed and registered clinicians in Ayurvedic medicine are present in the country. Manufacture and marketing of Ayurvedic drugs in the country is controlled by the Drugs and Cosmetics Act of 1940 [47].

In general two types of Ayurvedic drugs are available in the market/globally. The first type of drugs is part of the classical Ayurvedic formulations which follow the descriptions of Ayurved Samhita during its preparation and manufacture. The other is derived through patents and proprietary formulations made from herbal extracts [49]. There are currently 8403 pharmacies in India dispensing Ayurved drugs thus making India the largest consumer and exporter of Ayurved drugs in the world [47]. The Ayurvedic formulations and productions form one third of the total pharmaceutical business in the country and helps the nation in earning revenue of 4000 crores Indian rupees every year. However despite having such a large use of Ayurveda drug in India

the total number of adverse events reported is negligible when compared to that of alternative medicines and allopathy [47]. This is the same trend observed in other parts of the world resulting in the lack of side effect monitoring and reporting. Certain reasons due to this lack of Pharmacovigilance awareness in Ayurved system of medicine can be attributed to different factors like the safety methods that are used in this field of medicine have not evolved adequately and scientifically over time [47]. This can be further related to the fact that in none of the text books of Ayurveda, how to monitor and evaluate ayurvedic drugs has been mentioned. One example can be given in support of the above fact. Urmila et al. [46] interviewed a group of 80 Vaidyas or Ayurvedic physicians in India and found that 14 of the physicians refused to accept that there is any side effect associated with Ayurved and out of the remaining 66 clinicians, 48 responded by saying that they experienced some kind of adverse events in patients after Ayurved drugs were prescribed to patients. Out of these 48 clinicians only 14 of them reported the adverse events to the National Pharmacovigilance Centre in India thereby showcasing the fact that many of the trained Vaidyas actually lack the knowledge on safety aspects and perspectives related to these kinds of medicines. Also the increase of counterfeit drugs in the market and lack of quality manufacturers and manufacturing are questioning the safety of Ayurvedic drugs like never before. Understanding the pharmacokinetics and toxicokinetics of Ayurved drugs is also an area which has not been explored properly till date as most of the drugs are multi-ingredient in composition. Individualization of Ayurvedic medicine like Homeopathic medicine has also aggravated the lack of reporting of adverse events among patients [47].

Conclusion

Ayurvedic samhita and modern science = share almost the same principles of fetal growth and development. Many similarities between the two are observed like at the second month, the shape of the fetus decides its sex. However scientific principles behind this mechanism are still confounding. More than 5000 years back, Ayurvedic physicians wrote books and made conclusions according to their observations and knowledge without any scientific tools to support their ideas. Modern science today has all diagnostic laboratory equipments which were not available at that time, so the contribution of the Ayurvedic samhita is noticeable and paramount to any field of medicine as it can be opined that many of the basic and conceptual contexts of western or modern medicine that evolved at a much later stage are based on different traditional medicines in which Ayurvedic medicine has a very prominent position.

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Part XVII

Miscellaneous

Problems of Prematurity

45

Annie Abraham and C.S. Rejiya

Definition of Preterm or Premature

The average length of a normal pregnancy is 40 weeks (280 days) from the date of conception. Infants born before 37 weeks gestation are considered premature and may be at risk for complications.

Although, the rate of premature birth appears to vary by geographic region, the reported incidence varies between 6 and 10 %. Despite significant improvements in perinatal care, there has not been a concomitant reduction in the rate of premature births in developed countries. More than one out of every ten infants born in the United States is born prematurely. Advances in medical technology have made it possible for infants born as young as 23 weeks gestational age (17 weeks premature) to survive. These premature infants, however, are at higher risk for death or serious complications, which include heart defects, respiratory problems, blindness, and brain damage.

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Causes and Symptoms

The birth of a premature baby can be brought on by several different factors, including the following:

- Premature labor
- Placental abruption, in which the placenta detaches from the uterus
- Placenta previa, in which the placenta grows too low in the uterus
- Premature rupture of membranes, in which the amniotic sac is torn, causing the amniotic fluid to leak out
- Incompetent cervix, in which the cervix opens too soon
- Maternal toxemia or preeclampsia

Prematurity is much more common in pregnancy of multiples and for mothers who have a history of miscarriages or prior premature birth. Another identifiable cause of prematurity is drug abuse (e.g. cocaine) by the mother. Infants born prematurely may experience major complications due to their low birth weight and the immaturity of their organ systems. Some of the common problems among premature infants are **jaundice** (yellow discoloration of the skin and whites of the eyes), apnea (a long pause in breathing), and inability to breast or bottle feed. Body temperature, blood pressure, and heart rate may be difficult to regulate in premature infants. The

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lungs, digestive system, and nervous system (including the brain) are underdeveloped in premature babies and are particularly vulnerable to complications.

Short-Term Complications

In the first weeks, the complications of premature birth may include:

Breathing Problems

The primary function of the lung is gas exchange (i.e., they inhale oxygen and exhale carbon dioxide). Fetal breathing movements begin as early as 10 weeks of gestation, and the breathing of amniotic fluid in and out is essential for the stimulation of lung development. Fetal breathing movements tend to be erratic and occur only 30–40 % of the time up to 30 weeks of gestation. The failure of fetal breathing movements or a lack of amniotic fluid that can be breathed in and out results in underdeveloped lungs (i.e., pulmonary hypoplasia), which can be incompatible with extrauterine life. By approximately 30–32 weeks of gestation, the lungs make surfactant, a soap like substance that helps keep the air sacs (alveoli) open. Infants born before 28–30 weeks gestation lack alveoli and breath with their terminal bronchioles and primitive air sacs. After delivery, the breathing pattern generally becomes more regular and continuous, but immature regulatory systems can lead to brief episodes of not breathing.

Respiratory Distress Syndrome (RDS) is the most common problem in premature infants. Babies born too soon have immature lungs that have not developed surfactant, a protective film that helps air sacs in the lungs to stay open. With RDS, breathing is rapid and the center of the chest and rib cage pull inward with each breath. Extra oxygen can be supplied to the infant through tubes that fit into the nostrils of the nose or by placing the baby under an oxygen hood. In more serious cases, the baby may have to have a breathing tube inserted and receive air from a respirator or ventilator. A surfactant drug can be given in some cases. Extra oxygen may be needed for a few days or weeks. This condition primarily affects infants born before 35 weeks.

The chronic lung disease (CLD) that sometimes follows RDS in preterm infants born between 23 and 32 weeks, is also called bronchopulmonary dysplasia (BPD). BPD/CLD is a chronic disorder that results from inflammation, injury, and scarring of the airways and the alveoli. It is associated with growth, health, and neurodevelopmental problems during childhood. Positive-pressure ventilation, high oxygen concentrations, infection, and other inflammatory triggers all contribute to lung injury; but the primary cause of BPD/CLD is lung immaturity. Especially for infants born at less than 28–30 weeks of gestation, the lung tissue is very fragile and the injured lung tissue tends to trap air, collapse, or fill with mucus and other fluids, which further compromise lung growth and development.

Another complication of preterm birth is **apnea**, in which infants may stop breathing for 20 s or more, sometimes accompanied by a slow heart rate (bradycardia). Immaturity of the control of breathing is the major cause of apnea and bradycardia, although sometimes preterm infants have obstructive apnea (an obstruction to the movement of air in their airways). They require constant monitoring but generally respond quickly to stimulation (or in the case of obstructive apnea, repositioning). They may occasionally need to be given some positive-pressure breaths to get them breathing again. There is no agreement as to what constitutes pathologic apnea or the threshold of apnea that requires treatment.

Heart Problems

Preterm infants can experience a variety of cardiovascular disorders, ranging from major morphological defects to dysfunctional autoregulation of blood vessels (hypotension). By embryonic day 20, the cells that will form the heart begin to differentiate. The primitive heart beats by

4 weeks of gestation and is fully formed at the end of the sixth week. Because gas exchange occurs in the placenta, most of the fetal blood flow bypasses the lungs through the ductus arteriosus.

The most common heart problems premature babies experience are **Patent Ductus Arteriosus** (**PDA**) and low blood pressure (hypotension). PDA, which tends to affect babies born before 30 weeks, is a persistent opening between two major blood vessels leading from the heart. While this heart defect often closes on its own, left untreated it can cause too much blood to flow through the heart and cause heart failure as well as other complications. Low blood pressure may require adjustments in intravenous fluids, medicines, and sometimes blood transfusions.

Brain Problems

Babies born before 28 weeks are at risk of bleeding in the brain, known as an **Intra Ventricular Hemorrhage** (**IVH**). IVH is another serious complication of prematurity. It is a condition in which immature and fragile blood vessels within the brain burst and bleed into the hollow chambers (ventricles) normally reserved for cerebrospinal fluid and into the tissue surrounding them. Physicians grade the severity of IVH according to a scale of I through IV, with I being bleeding confined to a small area around the burst vessels and IV being an extensive collection of blood in the ventricles and in the brain tissue itself. Grades I and II are not uncommon, and the baby's body usually reabsorbs the blood with no ill effects. However, more severe IVH can result in **hydrocephalus**, a potentially fatal condition in which too much fluid collects in the ventricles, exerting increased pressure on the brain and causing the baby's head to expand abnormally. To drain fluid and relieve pressure on the brain, doctors either perform lumbar punctures, a procedure in which a needle is inserted into the spinal canal to drain fluid; install a reservoir, a tube that drains fluid from a ventricle and into an artificial chamber under or on top of the scalp; or install a ventricular shunt, a tube that drains fluid from the ventricles and into the abdomen, where it is reabsorbed by the body. Infants who are at high risk for IVH usually have an ultrasound taken of their brain in the first week after birth, followed by others if bleeding is detected. IVH cannot be prevented; however, close monitoring can ensure that procedures to reduce fluid in the brain are implemented quickly to minimize possible damage. Most hemorrhages are mild and resolve with little short-term impact. But some babies may have larger brain bleeding which causes permanent brain injury. Larger brain bleeds may lead to fluid accumulation in the brain (hydrocephalus) over a number of weeks. Some babies who develop hydrocephalus will require an operation to relieve the fluid accumulation.

Temperature Control Problems

Premature infants are not able to regulate their body temperatures as well as term infants. Factors that contribute to this problem are immaturity of the hypothalamic regulatory center, lack of subcutaneous fat (insulation shield), lack of brown fat (allows for thermogenesis in adverse climatic environmental conditions) and a relatively large body surface area to body mass ratio.

Typically, temperature control is maintained by providing an external heat source (radiant warmers or incubators/isolettes). These sources are set to provide heat to maintain a neutral thermal environment. In this environment, the infant has minimal energy expenditure to maintain core body temperature.

The majority of premature infants are able to regulate their body temperatures by a post conceptional age of 34 weeks. However, they remain at continued risk for poor thermal regulation at the extremes of environmental temperature. Parents should be appropriately counseled and encouraged to avoid subjecting their infant to temperature extremes.

Premature babies can lose body heat rapidly; they don't have the stored body fat of a full-term infant and they can't generate enough heat to counteract what's lost through the surface of their bodies. If body temperature dips too low,

hypothermia can result. Hypothermia in a preemie can lead to breathing problems and low blood sugar levels. In addition, a preemie may use up all of the energy gained from feedings just to stay warm, not to grow bigger. That's why smaller preemies require additional heat from a warmer or an incubator until they're larger and able to maintain their temperature without assistance. After you bring your baby home, you won't need to keep your house or apartment any warmer than you typically would.

Gastrointestinal Problems

The gastrointestinal (GI) tract digests and absorbs food, but it also has immune and endocrine functions and receives a good deal of input from the nervous system. It begins to form as early as the fourth week of gestation, and the stomach and the intestines are fully formed by 20 weeks of gestation. The intestines double in length in the last 15 weeks of gestation (to 275 cm at term). The intestinal absorptive cells form as early as 9 weeks of gestation, and endocrine and immune functions also begin early. Taste buds form at between 7 and 12 weeks of gestation. However, preterm infants have difficulty with digesting nutrients because many specialized cells are not fully functional.

The earliest coordinated reflexes are related to stimulation around the mouth, with mouth opening in response to perioral stimulation occurring at 9.5 weeks of gestation and head turning occurring by 11.5 weeks of gestation. The fetus swallows by 10–12 weeks of gestation and can suck by 20 weeks of gestation. After birth, the newborn's GI tract becomes colonized with bacteria, which aids with food digestion. Antibiotics alter this process.

Feeding intolerance is a common complication of preterm birth. The immature GI tract has difficulty digesting food necessary for ongoing growth and development. Very immature and sick infants receive parenteral (intravenous) nutrition with amino acids, glucose, electrolytes, and lipids. Preterm infants below 34–35 weeks of postmenstrual age require tube feeding because they cannot coordinate sucking, swallowing, or breathing. Providing the preterm infant with sufficient nutritional requirements for growth and development can complicate the treatment of other conditions.

Necrotizing Entero Colitis (NEC) is another complication of prematurity. In this condition, part of the baby's intestine is destroyed as a result of bacterial infection. In cases where only the innermost lining of the bowel dies, the infant's body can regenerate it over time; however, if the full thickness of a portion dies, it must be removed surgically and an opening (ostomy) must be made for the passage of wastes until the infant is healthy enough for the remaining ends to be sewn together. Because NEC is potentially fatal, doctors are quick to respond to its symptoms, which include lethargy, **vomitting**, a swollen and/or red abdomen, **fever**, and blood in the stool. Measures include taking the infant off mouth feedings and feeding him or her intravenously, administering **antibiotics**, and removing air and fluids from the digestive tract via a nasal tube. Approximately 70 % of NEC cases can be successfully treated without surgery. Premature babies who receive only breast milk have a much lower risk of developing NEC.

Gastro Esophageal Reflux (GER) is common in preterm and full-term infants, often presents as regurgitation, and may adversely affect growth and health. It may also be manifested by aspiration pneumonia, wheezing, or worsening of BPD/CLD because of an inability to protect the airway when refluxing. The presence of a nasogastric feeding tube increases the likelihood of reflux. Severe GER with aspiration of the stomach contents into the lungs is life threatening. GER is often treated with medications, including H2 blockers or protein pump inhibitors, which neutralize gastric acidity (and which may increase vulnerability to infection via the GI tract), and prokinetic compounds, which increase GI motility.

Blood Problems

Hematopoiesis is the generation of blood cells from stem cell progenitors. It begins in the

embryo 7 days after conception. Stem cells are active in the aortogonadomesonephron at 10 days and then shift to the liver and, finally, the bone marrow. There are developmental changes in the numbers and functions of hematopoietic stem cells and in the various differentiated blood cells (e.g., red blood cells, white blood cells, and platelets). Red blood cells in the fetus contain fetal hemoglobin, which is necessary for intrauterine gas exchange because it has a higher affinity for oxygen. Fetal hemoglobin levels decrease after birth.

Preemies are at risk of blood problems such as anemia and infant jaundice. Fetal blood loss, fetomaternal hemorrhage, and hemolysis can all result in congenital anemia, but the most common hematologic complication in preterm infants is anemia of prematurity. Anemia of prematurity is an exaggeration of the physiological anemia of infancy because of suppressed hematopoiesis for 6–12 weeks after birth and is earlier in onset and symptomatic. Its causes are multifactorial and include blood loss from frequent blood sampling, the shorter survival of red blood cells in preterm infants, a suboptimal response to anemia, and a greater need for red blood cells with growth. Preterm infants often need red blood cell transfusions, and many of the sickest and most immature infants need multiple transfusions.

Infant jaundice is a yellow discoloration in a newborn baby's skin and eyes that occurs because the baby's blood contains an excess of a yellow-colored pigment of red blood cells (bilirubin). Infant jaundice is common in babies born before 38 weeks.

Metabolic Problems

Premature babies often have problems with their metabolism. Some preemies may develop an abnormally low level of blood sugar (hypoglycemia). This can happen because preemies typically have smaller stores of glycogen (stored glucose) than do full-term babies and because preemies' immature livers have trouble producing glucose.

Problems of Immune System

Premature infants are at higher risk for infections. This risk is multifactorial. The primary source of immunity for the neonate is passively derived antibodies from the mother and this tends to occur primarily in the third trimester. Thus, the relative amount of antibody transferred is affected by the duration of gestation. Additionally, a significant proportion of premature infants who are hospitalized in intensive care units, require interventions such as IV therapy, and placement of central vascular catheters for providing nutrition, and invasive monitoring. All of these factors contribute to the increased risk of infections in this population. Premature infants present with nonspecific signs and symptoms of infection. This mandates close monitoring for infectious complications, both during hospitalization, in the immediate neonatal period, and in subsequent months during the first year of life.

Given their propensity for infections, the American Academy of Pediatrics recommends that all childhood immunizations be administered to premature infants at the appropriate chronological age. The only exception to this rule is the hepatitis B immunization, which should be initiated only after the infant's weight exceeds 2 kg. Despite lower titers of antibody response in these infants, there is no recommendation for additional doses of specific immunizations.

Passive prophylaxis for Respiratory Syncytial Virus (RSV) infection is currently recommended during the cooler winter months for certain premature infants at highest risk for serious complications from RSV. These guidelines are evolving. These infants will also benefit from receiving influenza immunization at 6 months chronological age during the cooler winter months (3).

An underdeveloped immune system, common in premature babies, can lead to infection. Infection in a premature baby can quickly spread to the bloodstream causing sepsis, a life-threatening complication. As a result, when a preemie's condition is getting worse, baby's doctor might check for an infection—even if there's no fever. Often, in such situations, baby may be given antibiotics until it's apparent that there's no infection.

Long-Term Complications

In the long term, premature birth may lead to these complications:

- **Cerebral palsy**. Cerebral palsy is a disorder of movement, muscle tone or posture that is caused by injury to a preemie's developing brain either during pregnancy or while the baby is still young and immature. Brain injury from poor circulation, insufficient oxygen supply, undernourishment or infection can all lead to cerebral palsy or other neurological problems.
- **Impaired cognitive skills**. Premature babies are more likely to lag behind their full-term counterparts on various developmental milestones. Upon school age, a child who was born prematurely might be more likely to have learning disabilities.
- **Vision problems**. Preemies born before 30 weeks may develop retinopathy of prematurity (ROP), a disease that occurs when blood vessels swell and overgrow in the light-sensitive layer of nerves at the back of the eye (retina). Sometimes the abnormal retinal vessels gradually scar the retina, pulling it out of position. When the retina is pulled away from the back of the eye it's called retinal detachment, a condition that, if undetected, can impair vision and cause blindness.

  Preterm infants are more likely than term infants to have significant abnormalities of all parts of the visual system, leading to reduced vision. The optic vesicles that will become the eyes form during the fifth and sixth weeks after conception. The eyeball is well formed by the lower limit of viability (22–25 weeks gestation). However, a pupillary membrane covers the anterior vascular capsule of the lens and gradually disappears between 27 and 34 weeks of gestation. The retina is a vascular layer in the back of the eye that translates light into electrical messages to the brain. The retina is the one of the last organs to be vascularized in the fetus. Blood vessel-forming cells originate near the optic disc (where the optic nerve enters the retina) from spindle cell precursors at 16 weeks of gestation and gradually spread across the surface of the retina, from the center to the periphery. Vessels cover only 70 % of the retina by 27 weeks of gestation, but in most cases the retina is completely vascularized to the nasal side by 36 weeks of gestation and to the temporal side by 40 weeks of gestation.

  The visual system functions very early, with the preterm infant blinking in response to bright light by 23–25 weeks of gestation and with papillary constriction in response to light by 29–30 of weeks gestation. By 30–32 weeks of postmenstrual age, the preterm infant begins to differentiate visual patterns. Visual acuity progressively improves with increasing postmenstrual age. The full-term neonate sees shapes (approximate visual acuity of 20/150) and colors and has a fixed focal length of 8 in. (anything closer or farther away becomes more blurry).
- **Hearing problems**. Premature babies are at increased risk of some degree of hearing loss. All babies will have their hearing checked before going home. The ear begins to develop at the end of 6 weeks of gestation and is fully developed by 20 weeks of gestation. A response to sound can be demonstrated in fetuses and infants born at 23 and 24 weeks of gestation, and auditory brainstem-evoked responses can be recorded this early in preterm infants. The shape of the waveform changes and the conduction time decreases with increasing gestational or postmenstrual age.

  One to two of 1,000 newborns suffer from congenital or perinatally acquired hearing disorders. The prevalence of neonatal hearing disorders has been reported to be increased 10- to 50-fold in infants at risk, which includes preterm infants. In addition to hearing impairment as a result of heredity, which is the cause of the largest percentage of hearing disorders, a number of in utero and neonatal complications (e.g., infections, immaturity, asphyxia, ototoxic medications, and hyperbilirubinemia) have been described to be risk factors for neonatal hearing disorders. Ventilated infants are at increased risk for otitis media.

Significant hearing impairment, often requiring hearing aids, occurs in 1–5 % of infants born at gestational ages of less than 25 or 26 weeks. Moderate to severe bilateral hearing impairment can distort the developing child's perception of speech and may interfere with his or her attempt at speech production. If the hearing impairment remains undetected through the critical period of language acquisition, that is, within the first 2 years of life, a profound impairment of receptive and expressive speech and language development can result. Early detection of hearing impairment facilitates early remediation (e.g., hearing aids or cochlear implants) and early intervention for speech and language acquisition. The prognosis for functional speech and language skills improves with the early detection and treatment of hearing impairment.

- **Dental problems**. Preemies who have been critically ill are at increased risk of developing dental problems, such as delayed tooth eruption, tooth discoloration and improperly aligned teeth.
- **Behavioral and psychological problems**. Children who experienced premature birth are more likely than full-term infants to have certain behavioral and psychological problems, such as attention deficit hyperactivity disorder, depression or generalized anxiety, and difficulties interacting with kids their own age.
- **Chronic health issues**. Premature babies are more likely to have chronic health issues—some of which may require hospital care—than are full-term infants. Infections, asthma and feeding problems are more likely to develop or persist. Premature infants are also at increased risk of Sudden Infant Death Syndrome (SIDS).

For some premature babies, difficulties may not appear until later in childhood or even adulthood. Not performing well in school is often a prime concern. Some studies suggest that premature babies may face an increased risk of type 2 diabetes and cardiovascular disease in adulthood.

The premature infant is ready for discharge when he/she is able to fulfill the following criteria: (1) ability to appropriately regulate their temperature without the need for technological support, (2) ability to ingest adequate calories to achieve consistent growth, and (3) to have demonstrated other parameters of global physiologic stability (the absence of clinically significant apnea, bradycardia, or hypoxemia). In addition, and most importantly, it is critical that the parents/caregivers feel comfortable with the care of the infant in the home environment. One of the issues that may alleviate some of the parental anxiety is training in infant CPR. Thus, the process of discharge of the infant is a continuum that begins several days to weeks prior to the actual discharge of the infant. Many of these infants will have additional needs and it is important that all of these needs and appropriate community resources are identified prior to discharge. At the time of discharge, the routine mandated screening for hearing and metabolic diseases should be completed with the results forwarded to the primary care physician.

Problems of Extreme Prematurity 46

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What Is Extreme Prematurity: Definition Incidence and Trend Consequences

- Embryology – which organ is at which stage of development and maturation at that moment both structurally and functionally?
- What is likely to be the problem with that state of specific development of that organ and that organ system?
- How will it affect the structural and functional development of growth and mayuration? Would there be any support required?
- What is the effect of the non mature or delay in maturation on the ultimate organ structure and function, and the impact on the individual immediately after birth, and morbidity and mortality due to this both in the immediate and in the long term?
  - Is there any disease which might be predisposed by this defective and/or incomplete and/or late organ maturation both structurally and functionally?
- Can it be prevented? If yes, how?
- Are there any advantages of extreme prematurity in the immediate or later adaptation to extrauterine life and final adaptation to the environment? If yes, what?

On 21st February 2007, Susan Donaldson James of ABC News presented an interesting perspective on premature babies and their issues of survival: Life at 21 weeks: Immature Lungs and a Handful of Fragile Skin and Pain – Last year, one of every eight babies born in the United States was premature, a number that is growing because of an increase the use of in vitro fertilization and other reproductive advances.

But the medical triumphs that allow a tiny 10-ounce baby to survive are often fraught with trauma and tragedy.

Consider this: At 25 weeks gestation or younger, a tiny baby can fit in the palm of a hand. Its skin is gelatinous – red and shiny and prone to infection like a burn victim – and sloughs off in the doctor's hand. The baby's windpipe is so small it can be crushed by a breathing tube.

And there is the pain, treated with narcotics that can cause dependency and withdrawal symptoms.

The long-term prognosis for babies this young includes mental retardation, blindness, deafness, cerebral palsy and – in the best scenario – learning disabilities, wrote Susan Donaldson James of ABC News on 21st Feb 2007

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What Is Extreme Prematurity? Definition

Preterm is defined as babies born alive before 37 completed weeks of pregnancy. The fetus is said to have attained viability if it has crossed 20th completed weeks of gestation and with the current level of neonatal care almost 50 % of such babies to be born after 24 completed weeks of gestation are likely to survive extra-uterine onslaught. Preterm is further subdivided depending upon the time of gestation in completed weeks post conception:

Extremely preterm – born before 28 completed weeks of gestation
Very preterm – born between 28 to 32 completed weeks of gestation
Moderate to Late preterm – more than 32 but less than 37 completed weeks of gestation [1].

Incidence

An estimated 15 million babies are born too early each year, which is more than one in ten babies, and about one million children die each year due to complications arising out of preterm birth. Many survivors face life time disabilities, including learning disabilities, visual and hearing problems. Globally, prematurity is the leading cause of death in children less than 5 years of age, and in almost all countries, incidence of prematurity is on the rise. In low socio-economic groups, 50 % of babies who are born at less than 32 completed weeks of gestation die due to lack of feasible and basic life care support, but in high income countries almost all the babies survive.

Why Does Preterm Birth Happen?

Most occur spontaneously, but in some cases, multiple births, chorio-amnionitis, other infections and chronic conditions, such as diabetes, high blood pressure, may result in preterm birth, but often the cause is not identified. Obesity or the low weight of the mother, vaginal infections, tobacco smoking, psychological stress and preeclampsia are important predisposing causes; few have suggested genetic predisposition and influence.

There are 3,519,100 preterm births in India in a year [2].

Preterm birth is one of the most common causes of death among infants worldwide [3]. About 15 million babies are preterm each year (5–18 % of all deliveries). In many countries rates of premature births have increased between the 1990s and 2010s [4]. Complications from preterm births resulted in 0.74 million deaths in 2013 down from 1.57 million in 1990 [5]. The chance of survival at less than 23 weeks is close to zero, while at 23 weeks it is 15 %, 24 weeks 55 % and 25 weeks about 80 % [6]. The chances of survival without long term difficulties is less [7].

Introduction

In recent years, excellent collaborations between the obstetrician and neonatologists and advances in neonatal care have made survival of pre-term neonates, especially extremely premature neonates possible [8]. Though preterm birth rates have increased all over the world, so has their survival especially in developed countries. Although most of the organs have been formed but their tertiary and terminal differentiation, for it to be structurally mature, is still pending in preterm babies. Moreover most organs are functionally, hence physiologically immature, which may or may not be entirely due to its anatomical and micro-structural immaturity. It is to be noted that the brain and lungs are the most susceptible among other organs, to the consequences of prematurity [9, 10]. Due to increasing specialization and cost of neonatal intensive care and the social and economic burden of disabilities on society, it is very important to justify intensive care and clinicians to be aware of the changing outcomes and the effect of long term disabilities and health problems on the survivors, their families and society as a whole. The focus should be on mortality and both short-term and long-term sequel of preterm birth, covering a broad range of

outcomes such as neuro-developmental, educational, behavioral, psycho-social, growth and health outcomes. The principal areas of concern are higher rates of temperature instability, respiratory distress, apnoea, hypoglycaemia, seizures, anaemia, jaundice, which can lead to kernicterus, feeding difficulties, visual and auditory impairment and peri-ventricular leukomalacia [11–14].

Respiratory System

Development Between months 3 and 6, the lung changes from pseudo-glandular to glandular; conducting epithelial tubes surrounded by thick mesenchyme are formed and there is extensive airway branching; after that the bronchioles are produced, the number of capillaries in close contact with the cuboidal epithelium increase and the alveolar epithelium begin to develop from about 16th week and completed by the 25th week, which is known as the canalicular stage. In this stage there is differentiation of pulmonary epithelium resulting in the formation of the future air-blood tissue barrier, and surfactant synthesis starts. Next is the saccular stage, which stretches from 24 to 40 weeks, where the alveolar ducts and air sacks are developed [15, 16]. Most peripheral airways form the widened airspaces, termed sacchules, which widen and lengthen the airspace, which is to become the future gas exchange region; this expands significantly [17, 18]. The fibroblastic cells undergo differentiation, producing extracellular matrix, collagen and elastin, which play a role in epithelial differentiation and control of surfactant secretion. This prenatal secretary lung epithelium has to drastically change to post natal absorptive epithelium, which is influenced by epinephrine, oxygen, glucocorticoids and thyroid hormone [19]. The role of Clara cells, along with ciliated and pulmonary neuro-endocrine cells that make up the epithelium of the bronchioles along the conducting airways, has also been indicated in regeneration after injury [20].

The Problem As the formation of terminal bronchioles and its maturation into sacchules is incomplete, so is the transition from secretary to absorptive epithelium; the lung fails to expel fluid, such that the transformation from a cystic space to pneumatic organ is hampered. The secretion of surfactant is insufficient, as only from the sixth month the alveolar type 2 cells starts secreting it. The amount is highly insufficient to inflate and keep inflated the fluid filled vesicles, the surface tension of which is huge compared to the meagre amount of surfactant being produced. So just after birth, respiratory distress starts, which has been termed as 'Infant Respiratory Distress Syndrome (IRDS/RDS)'. This can only be tackled by administration of artificial surfactant and not with glucocorticoid injection to the mother, as there are least number of alveolar type 2 cells mature enough to secrete it in response to the stimulation. Moreover, though it has been used for decades, meta-analysis shows that its use may be harmful, though it might be having some added advantages too [21–25]. Chronic lung disease is another problem which can develop if the baby survives the initial insult with artificial lung surfactant, specialized ventilation and controlled oxygenation via extracorporeal membrane oxygenation (ECMO), which was previously known as broncho-pulmonary dysplasia (BPD). Due to this respiratory insufficiency other organs also suffer as they too become victims of hypoxia, specially the brain and the hearing apparatus.

Respiratory Distress Syndrome and Bronchopulmonary Dysplasia

RDS is one of the most common lung disorders in premature infants, affecting about 10 %. Most infants who develop RDS show signs of breathing difficulty at birth or within the following few hours. The cause is largely the result of surfactant deficiency and lung immaturity in both structure and function. Many infants born with serious RDS go on to develop BPD. BPD usually develops within 1–2 weeks after birth; injury to small airways and microvascular development is implicated, the combined result of lung immaturity plus prolonged oxygen and mechanical ventilation. Infants with BPD are at risk for repeated pulmonary infections [26, 27].

Long Term Outcome

Respiratory Premature infants with a history of severe neonatal respiratory distress such as bronchopulmonary dysplasia may need more frequent follow-up to be vigilant for wheezing or pulmonary infections. Infants are likely to have persistent respiratory issues up to about 1 year of age. For example, hospitalization for a cold that causes wheezing and difficulty in breathing is not uncommon for infants who have had early respiratory difficulties. Respiratory difficulties tend to become less frequent after the first year; group daycare therefore may not be advisable in the first year. Infants with bronchopulmonary dysplasia can experience poor lung function until adolescence, when lung function typically becomes normal [28, 29].

Nervous System

Neurulation happens between 0 and 4 weeks, thereafter till the 12th week neuronal proliferation occurs, which is followed by neural migration, which continues till birth. Selective apoptosis and synaptogenesis starts within 17–18th week, continues till adolescence. Myelination starts after the 28th week and continues till adulthood [30, 31].

Results from multiple studies indicate that the effects of early life stress on the developing brain are significant and include, but are not limited to the following: increased amygdala volume, decreased activity in frontal cortical and limbic brain structures, and altered white matter structures.

Early life stress is believed to produce changes in brain development by interfering with neurogensis, synaptic production, and pruning of synapses and receptors. Interference with these processes could result in increased or decreased brain region volumes, potentially explaining the findings that early life stress is associated with increased amygdala volume and decreased anterior cingulate cortex volume [32].

Children born preterm are more likely to have white matter brain abnormalities early on causing higher risks of cognitive dysfunction [33]. White matter connectivity between the frontal and posterior brain regions are critical in learning to identify patterns in language [35]. Preterm children are at a greater risk for having poor connectivity between these areas leading to learning disabilities [36]. Neurological problems include apnea of prematurity, hypoxic-ischemic encephalopathy (HIE), retinopathy of prematurity (ROP), developmental disability, transient hyperammonemia of the newborn, cerebral palsy and intraventricular hemorrhage, the latter affecting 25 % of babies born preterm, usually before 32 weeks of pregnancy [34]. Mild brain bleeds usually leave no or few lasting complications, but severe bleeds often result in brain damage or even death [22]. Neurodevelopmental problems have been linked to lack of maternal thyroid hormones, at a time when their own thyroid is unable to meet postnatal needs [36].

Apnea of Prematurity (AOP) Apnea is the most common problem of ventilatory control in the premature infant, defined by a cessation of breathing for 20 s, or for a shorter period of time if accompanied by bradycardia, cyanosis, or pallor. The incidence and severity of apnea in premature infants are inversely related to gestational age. Approximately 50 % of VLBW infants require either pharmacologic intervention or ventilatory support. The peak incidence occurs between 5 and 7 days postnatal age. AOP usually resolves at 34–36 weeks postconceptual age [26, 27].

Apnea of prematurity tends to improve as the infant matures, usually resolving by about 42–44 weeks adjusted age [28, 29].

Retinopathy of prematurity (ROP) ROP occurs mainly in ELBW infants. Premature birth interrupts development of the retinal vasculature; prolonged exposure to supplemental oxygen required for the premature infant's lung function further exacerbates the condition. ROP often regresses or heals, but it can lead to severe visual impairment or blindness; myopia, amblyopia, or strabismus may occur [35].

Cerebral Palsy (CP) CP is an umbrella term describing non-progressive brain lesions that occur during early development, with resulting disorders varying by the week of gestation when the injury occurred. Preterm birth is only one of many risk factors for CP. The motor disorders of cerebral palsy may be accompanied by other disturbances including cognition, communication, perception, behavior and a seizure disorder [35, 37].

Developmental Disabilities As increasing numbers of VLBW and ELBW infants survive the early physical challenges of prematurity, attention is focusing on understanding longer-term implications for the child's developmental progress. Developmental disabilities resulting from premature birth range from cognitive and academic abilities, fine and gross motor skills, vision and hearing, and attention and behavioral deficits.

Long Term Outcome

Vision Visual problems, such as those associated with retinopathy of prematurity, should be followed regularly after discharge, with regular vision exams. Premature infants are also at increased risk for issues with eye muscle strength (e.g., strabismus) and may require glasses for correction [35].

Hearing All preterm infants should have their hearing tested at least once during their first year to make sure they do not have hearing problems. Early identification of hearing impairment is critical for the child's language development [35, 37].

Developmental Issues Ongoing follow-up physical examinations and monitoring of developmental milestones help identify cerebral palsy or other developmental problems as early as possible to enable an early start to appropriate interventions [35, 37].

Compared with full-term infants, premature infants have been found to demonstrate poorer scores relating to behavioral organization, attention to sensory stimuli, and self-regulation. It is thought that these early deficits are related to later deficits in children's attention regulation and executive functioning.

Numerous follow-up studies have been conducted on premature infants to assess later evidence of developmental delays:

- The EPICure Study followed a group of infants born at 25 or fewer weeks' gestation in 1995. More than 60 % received steroid treatment and 84 % received surfactant. At 30 months of age, 24 % of the survivors had severe disabilities in neuromotor, cognitive, hearing and vision domains, with cognitive impairment most common. These children were evaluated again at 6 years of age. Of the children observed to have severe disability at 30 months of age, 86 % still had moderate-to-severe disability at age 6. However, children with less severe disabilities at 30 months were developmentally comparable at age 6 to those without disability at 30 months [38].
- A study comparing rates of survival and neurodevelopmental impairment at corrected age 20 months in two groups of infants, one group born before the introduction of surfactant therapy vs. one group born after, found that overall survival rates increased from 49 % pre-surfactant to 67 % with surfactant therapy, but the rates of survival both without impairment and with impairment increased [39].
- A 12-year study completed in 1997 evaluated a group of premature infants born in pre-surfactant, transitional, and surfactant eras, and found that the rate of neurologic impairment remained constant at approximately 11 % [40].
- A 15-year follow-up study of children born at less than 29 weeks' gestation who received surfactant therapy found that a significant minority required intensive special education services through secondary school age [41].
- Presence of disabilities in more than one developmental domain predicts a more serious outcome for preterm infants. A follow-up study of children born <30 weeks' gestation evaluated neurological, motor, cognitive and behavioral effects. Thirty nine percent were

observed to have a normal developmental outcome, 17 % had a single disability, and 44 % had multiple disabilities. Multiple disabilities were associated with lower birth weight, BPD and continuing neuro-developmental difficulties as observed at age 2 [42].

- VLBW infants have a higher likelihood of having behavioral problems, delays in motor development, and difficulties in school, all issues that lie within the domain of executive functioning. The fact that preterm children show more generalized learning problems rather than specific learning disabilities (e.g., only a reading problem) supports the theory that preterm children's learning difficulties are caused by a global processing deficit rather than difficulties with particular skills [43].
- A study of 10-year-old VLBW preterm children showed that 20 % suffered from attention deficits compared with 8 % of full-term children [43].
- A study of 241 on children born between 22 and 25 weeks who were currently at school age found that 46 % had severe or moderate disabilities such as cerebral palsy, vision or hearing loss and learning problems. Thirty four percent were mildly disabled and 20 % had no disabilities, while 12 % had disabling cerebral palsy [44].

Gastrointestinal System

Gastrointestinal Immaturities Gastrointestinal and metabolic issues can arise from neonatal hypoglycemia, feeding difficulties, rickets of prematurity, hypocalcemia, inguinal hernia, and necrotizing enterocolitis (NEC). The "mechanical" immaturities of the premature infant's GI tract affect feeding tolerance, digestion, upper and lower GI motility and absorption. GERD and necrotizing enterocolitis are serious results of GI immaturity.

GERD – Both premature and full-term infants experience gastroesophageal reflux (GER), which is distinguished from GERD by the number and severity of reflux episodes and complications including strictures, malnutrition, respiratory disorders, and bleeding. For premature infants, an incompetent lower esophageal sphincter combined with delayed gastric emptying can lead to GERD. An indirect sign, especially in premature babies, is apnea associated with bradycardia.

Necrotizing enterocolitis (NEC) – occurs in about 10 % of premature infants weighing less than 1500 g. The pathogenesis of NEC is not completely established, but evidence suggests that it is a function of multiple causes including the presence of abnormal bacterial flora, intestinal ischemia, and intestinal mucosal immaturity/dysfunction. NEC is rare in infants who have not received feedings. When feedings are started and food moves into a weakened area of the intestinal tract, bacteria from the food can damage the intestinal tissues. Severe tissue damage can cause a perforation to develop, leading to infection in the abdomen. Initial signs of NEC include feeding intolerance, delayed gastric emptying and abdominal distension and tenderness.

Gastroesophageal Reflux Gastroesophageal reflux is common in premature infants and usually presents as spitting up after feeding. Severe GER can cause feeding difficulties, irritability, poor weight gain and respiratory problems. Keeping the infant in a semi-upright position after feeding may improve symptoms. GER tends to resolve as the infant's GI system matures.

Other Complications

- Hematologic complications include prematurity, thrombocytopenia, and hyperbilirubinemia (jaundice) that can lead to kernicterus.
- Infection, including sepsis, pneumonia, and urinary tract infection

Integumentary System

The basal layer of the epidermis becomes the stratum germinativum, which produces new cells that are displaced into the more superficial layers. By 11 weeks, cells from the stratum germinativum have formed an intermediate layer. Replacement of peridermal cells continues until

approximately the 21st week; thereafter, the periderm disappears and the stratum corneum forms. Proliferation of cells in the stratum germinativum also forms epidermal ridges, which extend into the developing dermis. These ridges begin to appear in embryos at 10 weeks and are permanently established by 17 weeks [45].

Late in the embryonic period, neural crest cells migrate into the mesenchyme of the developing dermis and differentiate into melanoblasts. Later these cells migrate to the dermoepidermal junction and differentiate into melanocytes. The differentiation of melanoblasts into melanocytes involves the formation of pigment granules. Wnt signaling regulates this process. Melanocytes appear in the developing skin at 40–50 days, immediately after the migration of neural crest cells. In white races, the cell bodies of melanocytes are usually confined to basal layers of the epidermis; however, their dendritic processes extend between the epidermal cells. Only a few melanin-containing cells are normally present in the dermis. The melanocytes begin producing melanin before birth and distribute it to the epidermal cells. Pigment formation can be observed prenatally in the epidermis of dark-skinned races; however, there is little evidence of such activity in light-skinned fetuses. Increased amounts of melanin are produced in response to ultraviolet light. The relative content of melanin inside the melanocytes accounts for the different colors of skin.

Anemia Anemia can be managed with iron supplementation. Supplemental iron should be given to preterm infants for 12–15 months.

Growth and Nutrition During the first 2 years, growth of the premature infant follows their age corrected for prematurity using a special premature growth chart. After the age of two, the child's progress may be followed using the standard growth chart.

Nutrient reserves in premature infants tend to be low at discharge, therefore nutritional status should be assessed at each follow-up visit. Ongoing supplements are recommended for preterm infants who are breastfed to meet additional nutritional needs. Formula-fed infants who gain weight well can transition from a preterm formula to a regular routine formula, while those who require additional calories to meet weight goals may be fed a discharge formula until they meet weight goals and can be transitioned to a routine formula.

Conclusion

Medical problems originating in the initial weeks of life may require care for months or years. Other conditions may be identified later in infancy or in childhood, therefore continual attentiveness to early signs is valuable.

Each follow-up appointment should include review of developmental milestones so that early intervention can be initiated if a developmental delay is identified or suspected. As with any developmental problem, early intervention and therapy is a key strategy.

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Sequencing of Naturally Aborted Human Foetuses: A Resource for New Knowledge

47

Samir K. Brahmachari

Human Genome Project since its inception in 1990 has raised many ethical issues. However, after the successful completion of Human Genome Sequencing in 2003, it was very clear that there exist significant variations in the genome of two individuals. The HapMap project [1] and the Indian Genome Variation Database [2, 3] led to many discoveries using which one could predict how individuals are prone to various diseases including drug response.

The advancement of technology has allowed us to determine the genome sequence of fetal DNA in the circulating blood of the pregnant mother. This has great medical implications especially with old age pregnancy where there is a higher risk of Down's Syndrome and other genetic disorders in the family. Sequencing the Unborn [4] has met with great triumph as well as protest. In this short commentary, I would like to focus on the idea of sequencing the naturally aborted fetuses and its potential biological implications. And it is believed that such a resource can easily be generated in India and such a database can provide answers to many crucial yet unsolved problems related to premature delivery to rejection of the fetus. This is also likely to answer several developmental issues that decide the progress from successful fertilization and fetal development to a fully mature baby.

Most powerful tool in genetics is to observe phenotypes by knocking out a gene. This has been very successful in smaller organisms and mammals like mouse. However, establishing a correlation between gene knock out and a phenotype has been successful for simple Mendelian genetic disorders but for complex disorders these are yet to be solved. Although large number of developmental genes and epigenetic modifications have been discovered through successful gene knockouts but application of this technique is not allowed for human embryos due to ethical reasons. Although recently discovered CRISPR-CAS technology [5] allows genome editing, the international scientific community has put major restrictions on the application of such technology for human embryo due to the uncertainty of their side effects resulting from wrong editing, which are yet to be deciphered. Recent human embryo editing by Chinese researchers [6] has raised many ethical concerns amongst the world's genomics community.

While we have been sequencing centenarians [7, 8] to understand the longevity and developmental genes that allows slow metabolism and slow decay of the cells and tissues, sequencing of naturally aborted fetus are like natural knockouts of the human genome. Sequencing of naturally

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aborted fetus will aid the discovery of deleterious mutations and rearrangement of genomes which will help discover the underlying principles of developments leading to fetal abortion.

The naturally aborted fetal genome sequencing will open up new possibilities of discovering the developmental errors that has finite likelihood to occur. While various chromosomal trisomy are not lethal at the fetal level it is likely that one will discover novel chromosomal rearrangements, duplications and deletions involved in development.

This database along with the genome sequence of premature born babies as in contrast to centenarian genomes will allow a comparative genomic analysis of fundamentally essential genes which are highly conserved and are highly sensitive to mutation, insertion/deletion, duplication etc.

The sequencing of naturally aborted human fetuses will aid in furthering our understanding of the underlying genetic factors behind miscarriages and reduce infant and maternal mortality.

The most crucial underpinning of this initiative would be that this will not result in any ethical issues. With its high frequency of population growth, India is likely to have higher number of naturally aborted fetuses. Thus India has a strategic advantage. However, it is important to remember that this project will not be successful for artificially aborted fetuses.

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Part XVIII

Ethics and Human Fetal Tissue Research

48 Ethics Pertaining to the Use of Aborted Human Tissues for Research and Therapeutic Purposes

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Introduction

With recent advancements in the field of medical science, tissue engineering, organ transplantation, human embryonic stem cell research and aborted fetal tissue transplants are becoming an attractive source for new scientific exploration and innovation in the field of medicine and healthcare. Human tissues can be used for transplantation studies and therapeutics as well as for in vitro purposes in understanding the simple and basic biology of different ailments as well as in the search for a better cure. However, like any other drug therapy or drug discovery process, tissue experimentation generates intensive debate in terms of ethics and law. Whether it is moral or immoral to use different aborted materials like embryonic stem cells from the inner cell mass or fetal tissues are discussed in this chapter, as these are the two primary materials that are obtained from aborted fetuses and are currently intense topics of discussion regarding their ethical use for experimental and therapeutic purposes.

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Religious Views regarding the Life of a Fetus and Its Abortion

Different religions have different views on when life begins in a fetus and therefore whether abortion should be allowed or not, and if it is allowed, under what circumstances or conditions. Most religions make an exception if the mother's health is in danger, but, beyond that, are uncompromising. Research on the growth and development of the fetus or on surgery of the unborn or fetal stem cells, may require aborted fetuses, and therefore it is important to understand religious beliefs. While one country may regularize abortion under certain circumstances including the right of the mother to have a choice in the matter, another country may forbid abortions on religious grounds. In Islam, abortion is normally regarded as wrong or *haram* (forbidden); however, under special circumstances abortion is permitted in

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order to save a mother's life. According to in Islam, abortion is permitted after 120 days of pregnancy under specific circumstances and some Islamic states even permit abortion after 16 weeks of the second trimester if it is thought that the pregnancy may cause major concern with regard to the mother's health and wellbeing or if the pregnancy outcome may result in a child with life long health issues [1].

According to the Jewish custom or Halacha a fetus becomes a fully fledged human when the head emerges from the womb at the time of birth. Before then the fetus is normally considered as a partial life [2]. The fetus to the Jewish community has a supreme value as it helps in forming a fully fledged life. Abortions are not permitted on the grounds of genetic anomalies; however, like in Islam, abortion is permitted only when it poses a great risk to the mother's life and health [2]. In Christianity, both the Catholic and the Orthodox church are against abortion. According to the Catholic Church, life is sacred and inviolable and it should not be hurt [4].

However, in ancient times, before the advent of the Jewish religion or Christianity or Islam, the understanding of when exactly life begins, was different. During the fourth century BCE to first century CE, Aristotle's theory of ensoulment was widely accepted in pagan Greece and Rome [3]. Aristotle taught that the fetus is originally a vegetable which evolves as an animal soul with further increase in gestation time. Ensoulment or the life of a human starts at 40 days after conception in case of males and 90 days for female fetuses according to this theory [3]. Therefore the idea of abortion was not condemned in the early stages of life as the fetus was believed to have a vegetable soul or an animal soul except in the later stages after ensoulment has occurred [3]. St. Augustine (354–430 CE) opined that a human soul can reside in an organized entity during early pregnancy; an abortion cannot be called a murder as no human soul is destroyed (only an animal or vegetable soul is destroyed) [3]. This was very much in accordance with Aristotle's philosophy regarding the abortion of a fetus. Much later, at the turn of the seventeenth century, the Greek concept was totally discarded (the religions mentioned earlier, in any case, viewed abortion as morally wrong, and the new theories provided a kind of justification); the concept of simultaneous animation gained momentum: it was surmized that the embryo acquires its soul right from the conception stage and not in the later weeks of gestation [3].

In Hinduism, abortion is generally viewed as a wrongdoing since it destroys an *atma* (soul). Like the Jewish and Muslim communities, Hindus permit the abortion process only when pregnancy poses a great risk to the mother. Normally, according to Hinduism, killing a fetus is like killing a priest, or it is even seen as worse than killing one's parents; in ancient times many would have lost their caste for committing this act. Many Hindus consider pregnancy as a duty since procreation is considered a duty to continue the family name, the caste and the community. Hindus also believe in re-incarnation, that is, when a person dies, his or her soul is recycled to this world in the form of a fetus in a different mother's womb; thus, in the eternal cycle of life, the fetus is held in high respect [5].

In contemporary times, however, like some other developing nations, India, which has a majority Hindu population with Muslims forming a large minority, has legalized abortion till a fixed number of weeks; this is to contain the population and for other reasons like the mother's health, the status of the fetus' health etc. At the same time, illegal abortions of female fetuses, known as 'female foeticide' also occur because of cultural preferences for male children [5].

Buddhism believes that harming the fetus is a murder or killing; but according to the views of the Dalai Lama, the Buddhist supremo who is remarkably modern in his thinking, while killing a fetus is wrong, depending on circumstances like whether the unborn child will be regressed and retarded from birth or create a serious problem for the parents, abortion can be implemented [4].

From the above discussion, it may be gathered, that an intense debate revolves around the issue as to whether the fetus can be considered as a life form, and this debate is largely fed by conceptual differences in various religions on when life begins, the sanctity of life and whether man has any right to kill or destroy God's creation. These arguments should also extend to man's

so-called claimed right to kill in wars or develop nuclear bombs that threaten mass killing. It almost appears that self-proclaimed defenders of the 'fundamentals' of many religions place less sanctity on the lives of innocent adults, who are at times slaughtered in the name of religion, at other times, in the pursuit of power and national interest, than on the innocent unborn, who has yet to emerge into life. Interestingly, debates on issues of power struggles, regime change, nuclearization and the threat of a nuclear war/disaster, which may involve mass killing of innocents, never seem to take a religious form, while the abortion debate and ethical issues surrounding fetal research are largely religious. Those who are against abortion believe that life starts at the time of conception, and those who favour abortion believe that abortion can be implemented only before the fetus is considered as a sufficient human and has the right to live [6].

According to the Catholic Church as discussed above, the embryo should be treated as a person right from its conception. It is believed that the fertilized egg during the conception period contains the full genetic code of a human being. The fertilization stage can be regarded as the beginning of a process that involves development and maturation and initiates the beginning of biological life. Some people also consider brain activity of the fetus as the first sign of introduction of life in the fetus, i.e., it is the time when the fetus starts to develop into a new individual human being. However, medical science has proved that the stage of brain activity in the fetus is more like a pre-conditioning phase where the fetal consciousness is actually not very well demonstrated [6].

Guidelines and Ethical Principles Pertaining to Aborted Material Research for Therapeutic Purposes

The advances in medical research and the scope for new ideas to develop into new life saving therapies cannot be denied. In fact, since the beginnings of medical science, particularly Western medicine, research has proved invaluable in developing new cures, and some of this research involved cadaveric as well as real life research. While emphasizing the need for experimentation, without which theory will remain theory, it should also be emphasized that certain principles, guidelines and ethics should be followed. Medical Councils of many countries have tried to frame ethical guidelines which suggest broad principles for empirical research. One such guideline is that of the Medical Research Council, UK, which suggests that human samples, be it cell lines or an aborted fetus, should be treated like donations [7]. Research pertaining to the use of human aborted samples and biological specimens should be treated with dignity, respect and transparency [7]. Cultural and religious differences play an important part while considering the use of aborted materials for research and medical purposes which researchers should be aware of. Therefore a connection of trust, belief and honesty should be developed between the researcher and the donor [8]. The Guideline also says that an important objective of research is to develop new products that will have less risk and optimum efficiency when compared to standard modes of therapy currently available in the market. Therefore the parameter of assessing the potential benefits and potential risks should be taken into serious consideration before aborted materials can be used for research or therapeutic purposes. Research, using aborted materials, should continue only when it is observed that the potential benefits outweigh the potential risks [9]. Researchers should also emphasize on the full use of existing collections rather than trying to accord new aborted material [8].

Another objective of research, according to the aforementioned Guideline is to know the unknown through trial and error methods. However, it suggests that special considerations should be followed while using human aborted materials by improving the awareness of the researcher with respect to the experiment or research or the therapeutic efficacy that certain trials or studies demand through different enriched and high quality journals and research materials for clinical datas and efficacy results; this can be easily accessed online so that there can be minimal waste of human and biological aborted samples [8].

Coming to the point of financial gains and transactions through selling or acquiring of

aborted human parts or the fetus, the Guideline notes that no such vested interests should be encouraging the donors, physicians, researchers and the recipients of such aborted therapies, nor should there be any conflict of interest [9].

The informed consent form is one of the best ethical practices to maintain transparency in any medical or biomedical research pertaining to the use of human aborted tissues and samples. It gives the donor a chance to verify information pertaining to the purpose and use of their samples, thereby giving them an honest chance to decide whether to donate or not [8]. Through a lucid and easily understandable 'Informed Consent Form' (ICF), donors get a first hand and detailed information whether their samples would be used for commercial use like therapeutic purposes or just for pure research. Literacy is another important aspect of taking informed consent from human donors. This is a very serious issue in many developing countries where illiterate or minimally educated donors can be coerced or foxed into donations of aborted tissues or fetus in lieu of personal financial and commercial gains of the researcher, clinician or the recipient of the tissue. The donor should be adequately accompanied by someone who can maintain transparency and not influence the donor's decision and in case if the donor is not able to apprehend the language or the content of the ICF, the person accompanying should explain the ICF to the donor [10]. Details of the process and the purpose of collecting the aborted material, where it will be stored, whether it will be given or sent to any other place for therapeutic purposes, and if used for transplantation studies, the methods by which it will be transplanted and for what medical condition, should be mentioned.

Ownership is another important concept while donating aborted materials for research and medical purposes. In case of fetal transplantations, aborted fetuses are normally used as therapeutics. While the fetus is inside the womb, the mother controls the ownership; however, once it has been donated after a proper informed consent process, the question remains whether the mother will have the same say as before or whether the ownership role of the mother will be diminished as the power of attorney of the aborted fetus will be transferred to the hands of the clinicians and the surgeons who will be conducting a fetal tissue transplantation or experimental procedures. It is a serious matter of debate and no straight forward answers are possible and the best way to ascertain such ownership or proprietary rights is to ascertain the situation on a case by case basis. There are other Guidelines like the world Health Organization's (WHO) International Ethical Guidelines for Biomedical Research involving Human Subjects and the Human Tissue Authority's (HTA) Codes of Practice, which are working on how best to conduct research in a fair manner without harming any community's sentiments or violating human or other rights or allowing unscrupulous persons to make financial gains instead of using aborted tissues for research that will ultimately benefit mankind [11]. It is useful to look into these well thought out but evolving guidelines as an aid to ethical research instead of dumping the baby with the bathwater in the rather intense debae on whether abortion or abortion research or fetal research should be permitted on ethical grounds. Contemporary state-of the-art research requires cell lines etc that may need the destruction of the embryo. If proper guidelines are followed and if a mother's right to choice is recognized, maybe some of the issues raised by religious and other bodies, may be circumnavigated.

Use of Human Embryonic Stem Cells from Pre Implanted Blastocyst Stage

One of the issues surround human embryonic stem cells which are an extremely attractive tool for cell therapeutic purposes and were first isolated by Thomson et al in 1998 at the University of Wisconsin, Madison [12]. These human embryonic stem cells (hES) as they are termed, are derived from the inner cell mass of the blastocyst stage of the embryos within 5 days of fertilization of the oocyte. hES cells are regarded as pluripotent stem cells as they can differentiate into any lineage of the ectoderm, mesoderm and

the endoderm except the extraembryonic tissues which forms the placenta [13]. Hence these cells are pluripotent but not totipotent stem cells. These hES cells are also immortal due to the presence of the telomerase gene, the protein product which ensures that the telomeric ends of the chromosomes are maintained after each cell division and does not undergo senescence [13]. The other cells which are pluripotent like hESC's are the human embryonal germ cells (hEG) derived from the primordial germ cell lines which would have formed the gametes ultimately if the fetus was not aborted. Most of the work pertaining to hESC and hEG are still in the pre clinical and trial phase and this has led to an intense debate over the use of such cells for therapeutic research, as isolation of these cells before the implantation stage requires an intervention and the termination of the pregnancy [13].

Therefore the status of the pre-implanted embryos is one of the most debated and sensitive issues, disputing on the isolation of the inner cell mass for the derivation of the viable human embryonic stem cells. However, it may be pointed out here that normally, from a medical point of view, pre-implanted embryos are mostly non viable due to 50–60 % of them being aneuploidies in nature [13]. Therefore selecting a non viable embryo for clinical and therapeutic purposes to treat different diseases does not collide with the ethical views of destroying a potential or viable life [13]. The debate surrounding the pre implanted embryo therefore is more of a moral and symbolic value; but in terms of research it has potential value for cure and the development of new therapies [13].

From the scientific point of view, an isolated inner cell mass (ICM) alone cannot give rise to a fetus; this is the misconception among many people especially in the non medical community who condemn the use of embryonic stem cell research. Trophoblast and extra-embryonic endoderm cells along with the ICM are required for proper implantation and nutrition of the embryo to help in the formation of the fetus. Thereby the ICM alone is a non viable material which cannot contribute to later stages of development [13].

Some others also opine that the cells of the inner cell mass are an important part of the embryo; however, when the ICM is cultured in vitro, the non viable cells perish, giving way to the viable cells of the ICM that can further divide into three lineages. This further strengthens the argument that the ICM may contain viable human embryonic stem cells that along with other constructs like the tropohoblastic layer and the extra embryonic endoderm could give rise to a fetus, but not alone by itself, and therefore the cells of the ICM only, can never be considered as an embryo [13].

Inspite of the above mentioned scientific observations and explanations, many opinions suggest that any part of the ICM can be considered as a viable tool for fetal development if the proper constructs were present, thereby depriving the chance of fetal development to the isolated ICM. These very people also share the opinion that any cell that can give rise to a fetus should be considered as an embryo, thereby contradicting scientific ideas, where an adult stem cell after a somatic cell nuclear transfer into an enuclated oocyte can give rise to an adult. In such cases, then, adult stem cells will be also regarded as embryos which very much refute the scientific observation and concepts pertaining to embryology [13]. Therefore the moral value of the non viable pre implanted embryos is much lesser than the viable pre implanted embryos; this concept needs further introspection [13]. The ban on embryonic stem cell research on flimsy unscientific grounds based on religious morality rather than on scientific perceptions and observations, appear a little confounding and not very justifiable [13].

There are two types of opinions regarding the developmental source and moral status of the pre-implantation embryo [13, 14]. Some view the embryo as a person and others see it as a non person devoid of any moral/rights status. Another view sandwiched between these two is the moderate view that the embryo needs protection as it can become a potential individual, and that it needs respect and is not to be played around with as a research material [13]. However, before the appearance of a primitive streak and the differentiation of

the three germ layers, embryos have a diversified and unpredictable function from where it can either give rise to twins or a normal individual, or abort; thus the embryo, at this stage, can be considered more like the units of a cell present in an adult system which together forms an organization called the tissue and performs a specialized function.

Also, ethically, human embryonic research is far more superior in terms of moral acceptance when compared to the use of aborted fetal tissue transplants because a fetus has a higher moral and symbolic value than a pre-implanted embryo, and secondly, because abortion of the fetus can often lead to pregnancy specific disorders to the mother [15].

Use of Fetal Tissue as a Research and Transplantation Material: Moral and Ethical Dilemmas

Modern research has indicated the use of fetal tissue as an attractive option for treating intractable diseases like diabetes, Parkinson's and various other neurological disorders. It is also an attractive option for preparing vaccines; the 1954 Nobel Prize was awarded for a polio vaccine that was developed from fetal kidney cells. Also, fetal cells were widely used for making vaccines for measles. On November 10, 1988 fetal tissue was used for the transplantation of fetal cells into a 52 year old suffering from Parkinson's disease at the University of Colorado Medical Centre [16]. More recently in India, Bhattacharya et al used fetal thymus tissue in a patient with arthritis.

Yet many bioethicists like Arthur Caplan believe that the very concept of using aborted fetal tissue for research and therapies is like a ticking time bomb of medical ethics [17].

But the possibilities of unveiling the mysteries of nature to provide cure for diseases that have plagued mankind for centuries through fetal tissue, outweigh outright rejection of the idea of fetal tissue research. Fetal tissue has some unique properties for transplantation and cell therapy purposes as it is hypo immune and can result in less rejection of the graft. It has a high proliferation and differentiation rate in vivo as seen from various pre clinical trials and medical therapies. However, the paucity of research has led to limited success, though the possibilities are immense. The best way to characterize fetal tissue research and transplantation till date is "Experimental" [18]. In the US, currently, 8 states prohibit the use of fetal tissue from dead fetuses and 17 states further prohibit the sale of tissue and fetal organs [19].

Current prominent and identifiable ethical concerns pertaining to the use of fetal tissues as a substance for biomedical and medical research are mainly based on the possibilities that a pregnant woman might be influenced upon to undergo abortion, or some women would be encouraged to get pregnant to induce abortion in exchange for financial gains. Thirdly, a woman's decision to donate an aborted fetus as a gift for human research and development may also be influenced by persuasion tactics or pressure [19]. The very concept of gift is flawed, because abortion is induced due to the non viability of a fetus or in other words, fetuses that suffer normal abortion, are not welcomed to join the human community. Therefore questions arise regarding how a non viable fetus can be considered as a gift [18]. The mother also cannot consider herself a donor of a gift because a gift has certain values whereas an aborted fetus does not have any such properties and is destined for closure. Therefore the very concept of donation or gift may itself be debatable.

However, there is evident truth in all these opinions. The role of a physician in influencing a mother's decision for fetus donation due to vested interests or financial gain, is not unknown, especially in developing countries where poverty-stricken pregnant mothers with many children are extremely vulnerable to exploitation.

Therefore, it is important to reiterate some of the points made earlier regarding the strict implementation of guidelines which are made after considerable consideration. These guidelines should also be not too rigid, allowing for flexibility; what is taboo today may be acceptable tomorrow. It must be remembered that in early

Christendom, cadavers were clandestinely dug up by scientists and artists who wished to study muscle structure, the veinous system etc; today, cadavers are used in medical schools all over the world for the study of human organs and systems. However, in the current context, certain measures may help maintain ethical standards: [19]

- Proper informed consent form, which is easily understood, and which contains all relevant information, is a must. Fostering and developing a relationship other than personal interests between the physician and the mother should be also encouraged. If the fetal tissue will be stored, it should also be mentioned in the ICF [19, 20].
- Health care practitioners who will be participating in the research should be in no way involved with the ICF process so as to avoid any conflict of interest. Also confidentiality of the donor should be maintained strictly [20].
- Ownership of the dead fetus or the fetal tissue, i.e., who will assume the proprietary or ownership rights, the mother, the physician or the recipients, should be clear [20].

Discussions

According to the World Medical Association (WMA) fetal tissues are not to be exploited, and should be provided according to the current guidelines and laws of the country, or international guidelines like the Declaration of Helsinki and the Declaration on Human Organ Transplantation should be followed [19]. The question that always rises up in case of fetal research and transplantation is that "Is it necessary to destroy a life for the sake of another life"? It is a highly ambiguous question that has no direct or correct answers because of the fact that a number of ethical, moral and scientific opinions are involved before coming to a general consensus. The same is the case for using human embryonic stem cells for therapeutic and clinical research. Under the circumstances, what is needed are strong independent Institutional Ethical Committees consisting of experts from diverse disciplines including law and the social sciences apart from subject experts, which can judge the merits of research on a case by case basis, and who are knowledgeable about their country's and international guidelines on medical research on human subjects, tissues or cells. The Ethics Committee must review the IFCs to safeguard the interests and health of the mother and the aborted fetus.

Conclusion

It is immensely important to understand what are the ethical dilemmas involved in aborted tissue research and whether this research is superior to the current approach towards cadaveric organ transplantation or xenotransplantation. The pros and cons of this kind of research have to be weighed. Modern medical theory speculates on the spectacular benefits of fetal tissue research and the potential cures of intractable diseases through the transplantation of cells derived from fetal tissues. On the other hand, such research can easily be exploited by doctors, donors, family members of donors, touts etc for personal financial gain. Further, one must also take into account the raging controversy on when life begins and whether abortion is ethical on religious grounds because it could mean the murder of an unborn human life, which also has a right to become a full human being at birth. Various national and international medical councils and other bodies have speculated on these issues and have come up with guidelines to prevent misuse of research and also take into account the mother's health and willingness to donate, thus also addressing religious and cultural issues. Perhaps, if strict surveillance is kept by ethical committees, which should bear in mind the long term benefits of research as well as the interests of all concerned, the new and novel ideas of medical scientific theorization can bear fruit in cures and therapies for humans in the future without harming the interests of the humans and unborn humans of the present.

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